UNIVERSITY OF ALBERTA

CHARACTERIZATION OF INDIGENOUS PLASMIDS IN THE *PASTEURELLACEAE* FOR USE IN GENETIC ANALYSIS

BY

LAURIE G. DIXON ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

DEPARTMENT OF MEDICAL MICROBIOLOGY AND INFECTIOUS DISEASES

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled CHARACTERIZATION OF INDIGENOUS PLASMIDS IN THE *PASTEURELLACEAE* FOR USE IN GENETIC ANALYSIS submitted by Laurie G. Dixon in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

There are no commercially available vectors for genetic analysis in the *Pasteurellaceae*. Given the importance of this family with respect to animal and human disease, a system for genetic analysis is needed. With this in mind, this study has characterized plasmids indigenous to the *Pasteurellaceae*.

Four small, broad-host-range antibiotic resistance plasmids that replicate within the *Pasteurellaceae* have been isolated: pLS88 and pHD148, from *Haemophilus ducreyi*, pHD8.1, from *Actinobacillus pleuropneumoniae*, and RSF0885, from *Haemophilus influenzae*. These plasmids can also replicate within the *Enterobacteriaceae*.

pHD8.1 and pHD148 are related to the IncQ plasmid RSF1010, which cannot replicate in the *Pasteurellaceae*. The three plasmids were shown to be incompatible. Sequencing of pHD148 and pHD8.1 showed that the area containing the RSF1010 *oriV* region was deleted in pHD148 and pHD8.1; however, *repA/B/C* genes were present and *oriV*-like regions were found elsewhere.

The plasmid pLS88 has been completely sequenced. Analysis of the antibiotic resistance genes revealed homology with RSF1010 and Tn*903*. The *ori* of pLS88 was small, and was independent of plasmid-encoded *rep* proteins. Given the size of the origin, pLS88 resembles the structure of narrow-host-range plasmids, but replicates as a broad-host-range plasmid.

The recombinant plasmids pDM2, derived from RSF0885, and pPW87/88, derived from pLS88, contain inserts from p2265 and pSa, respectively. These inserts were sequenced, and are linked with both transposon and integron containing structures which may be involved in recombination.

These plasmids contain features useful in the development of vector systems for genetic analysis within the *Pasteurellaceae*.

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LIST OF ABBREVIATIONS

amp	Ampicillin resistance gene
Am^r	Ampicillin resistance phenotype
Am^s	Ampicillin sensitive phenotype
AS	Amplification sequence
BAP	5% sheep's blood agar plate
BHI	Brain heart infusion (broth)
bla	β-lactamase gene; ampicillin resistance gene
bp	Base-pairs
CA	Chocolatized 5% sheep's blood agar plate
cat	Chloramphenicol acetyltransferase gene
cmp	Chloramphenicol resistance gene
Cm^r	Chloramphenicol resistance phenotype
Cm^s	Chloramphenicol sensitive phenotype
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
EDTA	(Ethylenedinitrilo)tetraacetic acid, disodium salt
EPB	Electroporation buffer: 0.3 M sucrose, 0.3 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 19% glycerol
EM	Electron microscope or electron microscopy
IHF	Integration host factor
In	Integron
Inc	Incompatibility group
IS	Insertion sequence

Kd	Kilodalton
kan	Kanamycin resistance gene
kb	Kilobase
Km^{r}	Kanamycin resistance phenotype
Km^{s}	Kanamycin sensitive phenotype
ORF	Open reading frame
ori	Origin of replication
oriT	Origin of conjugative transfer
oriV	Origin of vegetative replication for RSF1010
PAGE	Polyacrylamide gel electrophoresis
rep	Replication protein encoding gene
repA	RSF1010 gene encoding replication A protein; DNA helicase
repB	RSF1010 gene encoding replication B protein; DNA primase
repC	RSF1010 gene encoding replication C protein; DNA initiator protein
SDS	Sodium dodecyl sulfonate
Sm^{r}	Streptomycin resistance phenotype
Sm^{s}	Streptomycin sensitive phenotype
SSB	Single-stranded DNA binding protein
ssi	Single-stranded initiation sites; AT rich regions
strA	Streptomycin A resistance gene
strB	Streptomycin B resistance gene
sulI	Sulfonamide resistance gene, type I enzyme produced

sulII	Sulfonamide resistance gene, type II enzyme produced
Su^r	Sulfonamide resistance phenotype
Su^s	Sulfonamide sensitive phenotype
TBE	Tris-borate EDTA, pH 8.0 (5 x TBE is 0.45 M Tris base, 0.45 M boric acid, 0.01 M EDTA, pH 8.0)
TE	Tris-chloride EDTA, pH 8.0 (unless otherwise specified) 10 mM Tris buffer, 1 mM EDTA, pH with HCl
TEMED	N,N,N',N'-tetramethylethylenediamine
Tn	Transposon

I. INTRODUCTION AND LITERATURE REVIEW

A. THE *PASTEURELLACEAE*

1. BACTERIAL SPECIES WITHIN THE *PASTEURELLACEAE*

Phenotypic characterization and classification of fastidious bacteria like members of the *Haemophilus, Pasteurella,* and *Actinobacillus* groups has been difficult. Attempts to create genera and higher taxa in this area resulted in some highly artificial groupings. For example, in 1917 the Committee of the Society of American Bacteriologists on Characterization and Classification of Bacterial Types (Winslow, et al., 1917) proposed a genus *Haemophilus*, which included the small nonmotile parasites which grew only on media in the presence of blood or other body fluids. The influenza bacillus, the Koch-Weeks bacillus, the Ducrey bacillus, and the Bordet-Gengou bacillus were the main bacteria in this group. In 1981, in Bergey's Manual of Systematic Bacteriology, the family *Pasteurellaceae*, containing the genera *Pasteurella, Haemophilus,* and *Actinobacillus*, was proposed by Pohl and given status with the families *Enterobacteriaceae* and *Vibrionaceae* (Mannheim, 1984;

Mutters, et al., 1989).

The taxonomy of the family *Pasteurellaceae* as a whole and of its component genera is in some dispute, and has been examined by several methodologies. Major studies based on phenotypic traits include those on *Haemophilus* (Kilian, 1976; Broom & Sneath, 1981) and on *Actinobacillus* and *Pasteurella* (Sneath & Stevens, 1985). Advances in understanding the phylogeny of the members of the *Pasteurellaceae* have come from DNA-DNA hybridization studies (Mutters, et al., 1985; Pohl, 1981) and from rRNA-DNA hybridization studies (De Ley, et al., 1990). These studies have shown that the phylogenetic structure of the *Pasteurellaceae* is complex and that more than three genera are required to accomodate the diversity of species in this group. Because many species did not fall into defined clusters, the branching of genus-level clusters has remained unclear.

Recently, studies were undertaken to further clarify the phylogeny of this complex family through comparison of the full sequence of 16S rRNA in strains representing 54 described species (Dewhirst, et al., 1992). This method has proven extremely useful for determining phylogenetic relationships among other prokaryotic and eukaryotic organisms (Woese, 1987; Woese, et al., 1985*a*).

The 54 strains, composed of 15 *Pasteurella*, 16 *Actinobacillus*, and 23 *Haemophilus* organisms, fell into 5 clusters. Three of these clusters contained, as subsets, the sensu stricto genera *Haemophilus, Pasteurella,* and *Actinobacillus*. The fourth cluster was composed of 15 species from the above three genera, including *Haemophilus ducreyi, Actinobacillus pleuropneumoniae,* and *Pasteurella haemolytica*. The fifth cluster contained only *Pasteurella pneumotropica*, which did not fall into the *Pasteurella* main cluster in any of the analyses done (Dewhirst, et al., 1992). However, given the complexity of the branching seen during the study, no taxonomic changes were suggested.

The *Pasteurellaceae* is one of three families of gram-negative facultative anaerobes, many of which are pathogenic for humans and animals. Phylogenetically, these organisms belong to the gamma subgroup of the purple bacteria, clustering closely with the *Enterobacteriaceae* and the *Vibrionaceae* in 16S rRNA cataloguing studies (Woese, et al., 1985*b*). Members of the *Pasteurellaceae* are straight rigid, coccoid to rod-shaped, nonmotile, gram-negative cells, usually 0.2-0.3 x 0.3-2.0 μm in size. Pleomorphism with cell swelling and formation of filaments may occur. The *Pasteurellaceae* are fastidious, carbohydrate fermentating and anaerogenic. Complex media supplemented with yeast extract and serum or whole blood lysate are used for primary isolation. Varying patterns of nutritional requirements may include several amino acids, B vitamins, β-nicotinamide adenine nucleotides, and haematin or protoporphyrin (Mannheim, 1984). A list of the current members of the *Pasteurellaceae* and their natural hosts current members of the *Pasteurellaceae* and their natural hosts are found in Table 1.

Table 1. Human and animal species in the genera *Haemophilus, Actinobacillus,* and *Pasteurella*.

Human Species	Animal Species
H. influenzae	*H. equigenitalis* (horses)
H. parainfluenzae	*H. somnus* (cattle)
H. haemolyticus	*H. parasuis* (swine)
H. parahemolyticus	*H. paragallinarum* (poultry)
H. aphrophilus	*H. paracuniculus* (rabbits)
H. parahrophilus	*H. agni* (sheep)
H. paraphrohaemolyticus	*H. haemoglobinophilus* (dogs)
H. aegyptius	
H. segnis	
H. ducreyi	
Actinobacillus (Haemophilus) actinomycetemcomitans	A.*lignierisii* (cattle, sheep)
	A. equuli (horses, swine)
	A. suis (swine)
A. hominis	*A. capsulatis* (rabbits)
	Actinobacillus (Haemophilus) pleuropneumoniae (swine)
	A. muris (mice)
	A. rossi (cattle, swine)
	A. seminis (sheep)
	Actinobacillus (Haemophilus) avium (poultry)
P. multocida	*P. multocida* (cattle, poultry, sheep, swine)
P. belti	
Pasteurella (Actinobacillus) *ureae*	*Pasteurella (Actinobacillus) haemolytica* (cattle, sheep)
	Pasteurella (Actinobacillus) pneumotropica (rodents)
	P. gallinarum (poultry)
	P. aerogenes (swine)
	P. sp. new species 1 (called P. "gas") (dogs, cats)
	P. dogmatis (dogs, cats)
	P. gallinarum (poultry)
	P. canis (dogs, cattle)
	P. stomatis (dogs, cats)

P. avium (fowl)
P. volantium (fowl)
P. lymphangitidis (cattle)
P. mairi (swine)
P. testudinis (tortoises)
P. trehalosii (sheep)
P. species A (poultry)
P. species B (poultry)

2. *PASTEURELLACEAE*-MEDIATED DISEASE

In 1892, Pfeiffer identified a bacillus in the sputum of patients with influenza, claimed it as the etiological agent, and one year later isolated the bacterium on blood-containing media (Hirschmann & Everett, 1979). Doubts about its etiological importance resurfaced in the 1918-1919 pandemic when the bacillus could not be found in influenza victims, but in 1920 the bacillus was named *Haemophilus influenzae* (*Haemophilus* meaning "blood-loving"), reflecting Pfeiffer's claims (Hirschmann & Everett, 1979). In 1921, Thjotta and Avery found that growth of the bacillus depended on two factors, which they labelled X and V (Thjotta & Avery, 1921). In 1931, Pittman demonstrated immunological differences between capsulated and unencapsulated strains, and eventually discovered serotypes a-f (Pittman, 1931). During this time, it became clear that *H. influenzae* was an important cause of childhood bacterial meningitis and other infections, in both children and adults (Hirschmann & Everett, 1979).

Among the *Haemophilus* sp. that colonize man, *H. influenzae* is the most important from a clinical point of view (for review, see Hirschmann & Everett, 1979; Honig, et al., 1973). These infections can be divided into (a) acute, pyogenic and usually invasive infections in which *H. influenzae* is the primary pathogen, and (b) infections (usually chronic) in which *H. influenzae* is a secondary pathogen (Kilian, & Biberstein, 1984).

There are several human infections caused by *Haemophilus* species. Meningitis in children between 1 month and 2 years of age is most commonly caused by *H. influenzae*. Between the ages of 2 and 6 years, *H. influenzae* and *Neisseria meningitidis* occur with equal frequency. *H. influenzae* meningitis in children over 6 is uncommon, and in adults is usually seen as a complication of an underlying disease or condition, and results from the movement of the organism from a different area of the body. Such conditions include CSF leakage secondary to head trauma, chronic sinusitis and otitis media. Other diseases, such as diabetes, alcoholism, pneumonia and immunodeficiency states, including aging, may also predispose adults to *H. influenzae* meningitis (Jacobs, et al., 1983; Givner, et al., 1989; Greene, 1978).

H. influenzae is the most common cause of epiglottitis. This infection is usually acute, with an abrupt onset of obstructive laryngeal edema. The disease is most frequently seen in children between 2 and 4 years of age, with very few cases in adults (Jones, et al., 1976).

Acute otitis media is usually caused by either *Streptococcus pneumoniae* or *H. influenzae* in children between the ages of 6 months and 2 years. *H. influenzae* strains may also be significant causes of otitis media in adolescents and adults. Concomitant bacteremia and/or meningitis occurs in about 4% of cases in children (Dajani, et al., 1979; Wald, 1989).

Acute sinusitis is most frequently caused by either *S. pneumoniae* or *H. influenzae*. There is evidence that these organisms may be secondary invaders following viral sinusitis (for example, rhinovirus infection [Murphy & Apicella, 1987]). *H. influenzae* does not play a role in chronic sinusitis (Hirschmann & Everett, 1979; Murphy & Apicella, 1987).

Chronic bronchitis is an ill-defined clinical entity characterized by a persistent, productive cough, wheezing and shortness of breath. *H. influenzae* is recovered from about 60% of patients. Because of the normal colonization of the upper respiratory tract by the organism, the role of *H. influenzae* in chronic bronchitis is unclear. Experimental data suggests a pathogenic role for *H. influenzae*, probably in combination with other host factors, including immune status or other pulmonary infection (Hirschmann & Everett, 1979; Murphy & Apicella, 1987).

Bacteremia is a frequent manifestation of *H. influenzae* (mostly serotype b) infection, and may result in several other clinical manifestations of infection. Seeding of the soft tissues results in cellulitis; septic arthritis and osteomyelitis may also complicate bacteremia. Neonatal sepsis caused by *H. influenzae* has been described, resulting from perinatal transmission (Dajani, et al., 1979; Friesen & Cho, 1986; Holmes & Kozinin, 1983; Wallace, et al., 1983).

H. influenzae pneumonia can be another manifestation of systemic infection (such as meningitis, epiglottitis, bacteremia or otitis media), or can develop as a complication of a respiratory tract infection (such as chronic bronchitis or acute tracheobronchitis). Definitive diagnosis may be hampered by the presence of the organism in the upper respiratory tract in healthy individuals. Definitive diagnosis may require growth of the organism from blood, pleural fluid or lung aspirate (Honig, et al., 1973; Holmes & Kozinin, 1983).

Haemophilus-mediated endocarditis is most often due to *H. aphrophilus, H. paraphrophilus* or *H. parainfluenzae*; *H. influenzae* is infrequently isolated from such patients. Incidence is highest in young to middle-aged adults and mortality is usually about 10% to 15%. Higher rates (25% to 50%) may reflect difficulty in isolating these fastidious organisms from blood cultures, combined with inappropriate or inadequate antimicrobial therapy (Chunn, et al., 1978; Lynn, et al., 1977; Parker, et al., 1983).

Haemophilus species, particularly *H. influenzae* and *H.*

parainfluenzae have been reported as possible causes of nongonococcal urethritis, female genital tract infections, postpartum bacteremia and neonatal sepsis. In one study, 10% of nongonococcal urethritis cases investigated recovered *H. influenzae* or *H. parainfluenzae*: these organisms were not isolated from asymptomatic men (Sturm, 1986). Other studies have demonstrated the recovery of *H. influenzae* from blood cultures of women with postpartum bacteremia and neonates with bacteremia or meningitis (Wallace, et al., 1983; quentin, et al., 1989). Other sites from which *H. influenzae* has been cultivated include the vagina, endometrium, cervix, Bartholin's glands, fallopian tubes, male urethra, prostatic fluid, and fetal tissues (Sturm, 1986; Wallace, et al., 1983; Chowdury & Parek, 1983; Quentin, et al., 1989; Paavonen, et al., 1985). Genital tract infections associated with *Haemophilus* species can be linked to the presence of intrauterine devices, suggesting that *Haemophilus* species may behave as opportunistic agents in these areas (Paavonen, et al., 1985; Wallace, 1983).

H. aegyptius and *H. influenzae* have long been known to cause seasonal epidemics of acute purulent conjunctivitis, also referred to as pink eye (Albritton, 1982; Harrison, et al., 1989; Harrison & Broome, 1989). Localized outbreaks of acute conjunctivitis occurs among persons who share towels, handkerchiefs, or other objects that come in direct contact with the skin of the face or eyes. The pink color of the sclera and the presence of a serous or purulent discharge is virtually diagnostic of *Haemophilus*-mediated conjunctivitis (Harrison, et al., 1989; Leibowitz, et al., 1976; Ingham & Turk, 1969; Gigliotti, et al., 1981).

Brazilian purpuric fever (BPF) is a recently recognized pediatric infectious disease caused by a single clone of *H. aegyptius*. Since its first description in 1985, researchers have sought to determine the basis for the invasiveness of an organism previously responsible only for purulent conjunctivitis. The disease is characterized by fever, abdominal pain and vomiting, hemorrhagic skin lesions, vascular collapse, and death (Brazilian Purpuric Fever Study Group, 1987; Harrison, et al., 1989a; 1989b; Swaminathan, et al., 1989; Carlone, et al., 1989). This phenomenom was thought to be localized to Brazil, but in 1986 a child in Australia was reported to have the symptoms of BPF (Wild, et al., 1989). The virulence factors of the BPF clone and the pathogenesis of BPF are now undergoing further study, and surveillance has been established in BPF-endemic areas to monitor the disease and the spread of the implicated organism.

The first differentiation of the sexually transmitted disease chancroid (also called soft chancre) from syphilis was made by Bassereau in France in 1852, when he demonstrated that patients with chancroid could be reinfected at other skin sites by autoinoculation of purulent material from the ulcer (Morse, 1989). In 1889, Ducrey at the University of Naples published data on the identification of the "virus" of the soft chancre. At weekly intervals, he inoculated a new skin site with material from recent ulcers, and could maintain ulcers for as long as 15 generations. In every patient, he found a single organism in the ulcer exudate which he described as a "short, compact, streptobacillary rod with rounded ends" (Morse, 1989). Successful cultivation of the bacillus did not occur until the late 1890's (Davis, 1903). Later reseachers were able to produce disease in such animals as apes, chimpanzees, humans and rabbits (Morse, 1989). Named *Coccobacillus ducreyi* by Neveu-Lemaire in 1920, it was not renamed *Haemophilus ducreyi* until several years later (Kilian & Biberstein, 1984).

Chancroid is a highly contagious sexually transmitted disease characterized by painful genital ulcers and tender inguinal lymphadenopathy. The disease is worldwide in distribution and is endemic in South America, Africa, East and Southeast Asia and India. Sporadic outbreaks of chancroid have been reported in the United States and Canada (Morse, 1989). These outbreaks have occurred almost exclusively among heterosexuals and, frequently have been traced to female prostitutes (Schmid, et al., 1987).

Recently, an association between *H. ducreyi* infection and human immunodeficiency virus (HIV-1 and HIV-2) has been observed (Kreiss, et al., 1986). Genital ulcers due to chancroid and other agents (such as syphilis) apparently facilitate the passage of the virus between sexual partners (Kreiss, et al., 1986).

Haemophilus species other than *H. influenzae*, *H. aegyptius* and *H. ducreyi* are uncommon agents of human infection. As members of the normal upper respiratory tract and oral cavity flora, organisms such as *H. parainfluenzae, H. aphrophilus* and *H. paraphrophilus* are sometimes isolated from bronchitis, sinusitis, otitis media, pneumonia, bacteremia and endocarditis, and rarely from meningitis and brain abcess. *H. haemolyticus, H. parahaemolyticus* and *H. paraphrophaemolyticus* are also normal flora of the respiratory tract, and are rarely isolated from human infection (Jawetz, et al., 1987a; Kilian & Biberstein, 1984).

Most members of the genus *Actinobacillus* are found both as pathogens and as commensal organisms in domestic animals. Occasionally they may be found associated with disease in man, and one species, *A. actinomycetemcomitans*, recently recommended to be reclassified as *Haemophilus actinomycetemcomitans* (Chuba, et al., 1988; De Ley, et al., 1990; Dewhirst, et al., 1992), occurs only in man. As normal flora, *Actinobacillus* species are to be found in the alimentary, respiratory and genital tracts of animals, and the change from a commensal to a pathogenic organism usually requires some factor to assist the entry and establishment in tissue; in other words, *Actinobacillus* species are opportunistic pathogens (Phillips, 1984).

A. actinomycetemcomitans is associated with endocarditis, bacteremia, wound infection and dental infections. Although the organism has been frequently co-isolated with *Actinomyces israelii* from dental abcesses, it has also been isolated from similar abcesses without the actinomycetes (Phillips, 1984). The most common infection associated with *A. actinomycetemcomitans* is subacute bacterial endocarditis, most frequently in individuals with valvular damage due to congenital heart diseases (Kaplan, et al., 1989; Kristinsson, et al., 1988; Wilson, 1989). Prosthetic mitral valve and aortic valve endocarditis have also been reported (Grace, et al., 1988; Wilson, 1989).

Pasteurella species are primarily animal pathogens, but they can produce a range of human diseases. *P. multocida* occurs worldwide in the respiratory and gastrointestinal tracts of a wide variety of domestic and wild animals. It is commonly associated with dog and cat bite wound infections in humans (Carter, 1984; Jawetz, et al., 1987b). *P. multocida* may also be isolated from the respiratory tract, where it may exist as a commensal or as a cause of infection; in most cases, patients have a history of occupational or recreational exposure to animals (Carter, 1984; Starkebaum & Plords, 1977; Weber, et al., 1984). Systemic infections due to *P. multocida* in humans are rare, and patients with systemic infection usually have past or current exposure to animals, in most cases cats and/or dogs (Carter, 1984; Weber, et al., 1984).

Most of the bacterial species within the *Pasteurellaceae* form part of the normal flora of both wild and domestic animals (see Table 1), and are opportunistic pathogens in these animals (Carter, 1984; Kilian & Biberstein, 1984; Phillips, 1984). Consequently, many members of the *Pasteurellaceae* are of economic importance, causing disease in cattle, swine, sheep and poultry.

B. PLASMIDS FOR GENETIC ANALYSIS

1. INTRODUCTION

The discovery of plasmids occurred in Japan, with the appearance of multiple antibiotic resistant *Shigella* strains in the early 1950's, and the rapid increase in the number of these strains attracted the attention of microbiologists. In 1959 reports indicated that multiple resistance was transferable by mixed cultivation between antibiotic-resistant and -sensitive *Shigella* and *E. coli* strains. In early 1960, it was confirmed that the transfer of multiple antibiotic resistance was not mediated by bacteriophages, deoxyribonucleic acids, or other filterable agents. At the same time, it was proven that antibiotic resistance was transmitted during mixed cultivation between F- or Hfr *E. coli* strains and sensitive *Shigella*, regardless of the F agent polarity, indicating that transferable antibiotic resistance was transmitted independently of chromosomal transmission from donor to recipient cells, and that this agent is different from the F factor. Later that year, the spontaneous loss of antibiotic resistance was observed in bacteria during storage in a cooked meat medium. In addition, transmission of antibiotic resistance was interrupted when a mixed culture was subjected to blender treatment, indicating that transfer was mediated by conjugation, and that the antibiotic resistant agent was independent of the bacterial chromosome. Incubation of a mixed culture resulted in the rapid acquisition of multiple antibiotic resistance by a majority of the recipient cells, suggesting that transmissible antibiotic resistance is a property that replicates at a faster rate than the bacterial chromosome. The term "R-factor" was proposed that year for the property of transmissible antibiotic resistance (Mitsuhashi, 1977). Since that time, R-factors, also called plasmids, have been extensively studied and characterized.

Plasmids are extrachromosomal genetic elements found in a wide variety of bacterial species. They are usually circular, double stranded DNA molecules that range in size from 1 kb to more than 200 kb. They behave as accessory genetic units, usually dispensible, that replicate and are inherited independently of the bacterial chromosome.

Plasmids represent an important factor in bacterial evolution: they enable rapid, short-term adaptation of bacteria to changing environmental conditions; they confer gene amplification; and they can be transferred within one or between many species. Normally, plasmids are not essential to their hosts: however, they often contain genes coding for enzymes that, under certain situations, are advantageous to the bacterial host. Among the phenotypes conferred are resistance to antibiotics; production of antibiotics; degradation of organic compounds; and the production of colicins, enterotoxins, and restriction and modification enzymes.

Plasmids are particularly useful as tools for genetic analysis. Plasmid genes are easier to work with than genes located on the chromosome, whose location and function can be obscured within the total genetic content of the bacterial genome. A plasmid that carries a set of mobilizing and transfer genes that promote bacterial conjugation is called a conjugative plasmid, and a plasmid is called relaxed if it is present in a multicopy form in the host cell (Krishnapillai, 1988). Two plasmids that cannot coexist in one host cell in the absence of selective pressures are considered to be incompatible, or belonging to different incompatibility groups (Couturier, et al., 1988).

The control mechanisms determining replication, copy number, and plasmid incompatibility are usually plasmid encoded and involve the initiation of replication (Kolter & Helinski, 1979; Nordström, 1985; Novick, 1987; Scott, 1984; Thomas, 1988). In addition to plasmid-inherited determinants, replication initiation is dependent on various host-encoded enzymes (Scott, 1984). Many plasmids can therefore replicate only in one or a few closely related hosts. Promiscuous plasmids encode more of their own replication enzymes, and therefore are adapted to a wide range of bacteria and can be inherited in a variety of hosts.

One of the most interesting discoveries in microbiology since the introduction of antibiotics for treating bacterial infections has been the emergence and spread of resistance plasmids, which have also been important for studies in molecular and microbial genetics. Although some bacterial clones have a resistance to antibiotics in the absence of exposure, most bacteria acquire resistance due to the acquisition of plasmids encoding proteins which allow the bacteria to export, inactivate, or avoid the antibacterial action of the antibiotic. These antibiotic resistance genes are the most commonly used selectable markers, and allow for the easy selection of successfully transformed bacteria.

The most commonly used antibiotic gene markers include ampicillin, tetracycline, chloramphenicol, and aminoglycoside (eg. kanamycin and streptomycin) resistance genes (Sambrook, et al., 1989). These antibiotics and their plasmid-mediated resistance mechanisms each operate differently. An example of each kind of antibiotic resistance is given below:

a) Ampicillin binds to and inhibits a number of enzymes that are involved in cell wall synthesis. The usual mechanism of plasmid-mediated ampicillin resistance involves the production of an enzyme that is secreted into the periplasmic space and catalyzes the hydrolysis of the β-lactam ring (Sambrook, et al., 1989). Chromosomally mediated ampicillin resistance due to alterations in penicillin binding proteins has not yet been associated with plasmids (Bush, 1989*a*, 1989*b*, 1989*c*, Halbert, 1988).

b) Tetracycline binds to a protein of the 30S ribosomal subunit and inhibits ribosomal translocation (Sambrook, et al., 1989). One plasmid-mediated tetracycline resistance mechanism involves an inducible tetracycline efflux protein which transports the antibiotic out of the bacterial cell (Izaki, et al., 1966; McMurry, et al., 1980; Kleckner, 1989).

c) Chloramphenicol binds to the 50S ribosomal subunit and inhibits peptidyl transferase so that ribosomal translocation and peptide bond formation are uncoupled (Weinstein, 1975). The plasmid-mediated chloramphenicol resistance gene encodes a protein acetyltransferase that catalyzes the formation of hydroxyl acetoxy derivatives of the antibiotic which are unable to bind to ribosomes (Shaw, 1984).

d) Kanamycin is a deoxystreptamine aminoglycoside that binds to the 30S ribosomal subunit and causes the misreading of mRNA. One plasmid-mediated method of inactivation is *via* production of an aminoglycoside phosphotransferase which phosphorylates the antibiotic and prevents its entry into the bacterial cell (Sambrook, et al., 1989).

e) Streptomycin is also an aminoglycoside that binds to the 30S ribosomal subunit and prevents the ribosome from binding to mRNA (Phillips & Shannon, 1984). An example of a plasmid encoded enzyme is, again, an aminoglycoside phosphotransferase (for example, the RSF1010 streptomycin resistance gene) which phosphorylates the antibiotic (Phillips & Shannon, 1984).

There are several plasmids which also carry sulfonamide or linked sulfonamide-streptomycin resistance genes. Sulfonamides are competative inhibitors of dihydropteroate synthase, an enzyme in the bacterial biosynthetic pathway producing tetrahydrofolic acid (the single carbon donor in the production of amino acids and nucleic acids) from its substrate para-aminobenzoic acid. The plasmid encoded sulfonamide resistance protein is a dihydropteroate synthase which is resistant to sulfonamide competition with its normal substrate (Willson, 1989).

Virtually all plasmid vectors in use carry one or more of the above mentioned antibiotic resistance genes for selection of transformed bacteria. Other selectable markers include the use of the *lac* operon, which allows differentiation of parental *vs*. recombinant plasmids by the production (or lack of, respectively) of an active β-galactosidase (Ullman, et al., 1967) Plasmid selection by insertional inactivation of a lethal gene, such as colicin E3 inactivation (Vernet, et al., 1985), is also a viable selection method. There are several other selection methods currently in use for DNA cloning strategies.

Plasmids to be used in cloning must encode information for replication and partitioning in daughter cells after bacterial fission. The host range must include both the bacterial species of interest, and, in general, *E. coli*, because of its wide use in genetic analysis. The vector must have a marker, usually an antibiotic resistance gene, that allows for easy selection of bacteria containing the vector. Several attributes that make a vector useful for cloning include:

a) should be capable of autonomous, relaxed replication, even when covalently joined to foreign DNA.

b) should have one or more markers for selection of bacteria containing recombinant vectors.

c) should contain a cloning site in a region not essential for replication, preferably within a marker and with a second marker for counterselection, for the enzymatic insertion of foreign DNA.

d) should be small in size, and be easily separated from bacterial nucleic acids and purified.

Under normal conditions, many plasmids can be transmitted to new hosts through bacterial conjugation. However, plasmid vectors commonly used lack genes required for transfer and/or mobilization and cannot effect their own conjugal transfer. Some of these plasmids do contain an origin of transfer (*oriT*) and can be mobilized by a conjugative plasmid which supplies the missing gene products in *trans* (Araki, et al., 1987). Plasmids can also be introduced into bacteria *via* transformation and transduction. Some bacteria, such as *H. influenzae*, are naturally competent, and will import plasmid DNA from outside of the cell, if the plasmid contains a specific uptake sequence (Chung, et al., 1989), but others, such as *E. coli*, require high levels of $CaCl_2$ (Cohen, et al., 1972) or other membrane active components, such as DMSO, reducing agents, and hexaminecobalt chloride (Weston, et al., 1981). A newer method of transformation, electroporation, involves the introduction of DNA into cells by applying a high voltage pulse, which is thought to create holes in the bacterial membrane, thus allowing DNA into the cell, and is useful for prokaryotes lacking natural gene transfer systems (Dower, et al., 1988; Miller, et al., 1988; Dower, 1990).

2. INC Q-LIKE PLASMID VECTORS

The incompatibility group Inc Q (also referred to as Inc P4) consists of a group of plasmids characterized by their relatively small size, medium range copy number (typically 15-

30 copies per bacterial cell), and broad-host-range replication system. These plasmids are typically isolated from such gram-negative bacilli as *Salmonella* spp., *Proteus* spp., and *Pseudomonas* spp., and usually confer resistance to streptomycin and the sulfonamides. Other resistance determinants have been found in a small number of known Inc Q plasmids. These plasmids are mostly nonconjugative, but can be mobilized into a wide variety of bacterial species if a conjugative helper plasmid is present (Frey & Bagdasarian, 1989).

Replicons in this incompatibility group have been used in the development of vectors for genetic analysis, and have been especially useful in those gram-negative bacteria inaccessible to the classic *E. coli* cloning systems based on Col E1 type replicons or *E. coli* specific bacteriophages. These recombinant vectors have been particularly useful in the analysis of *Pseudomonas* spp. (Frey & Bagdasarian, 1989), as well as in promoting plasmid transfer to plants (Frey & Bagdasarian, 1989).

Recently, certain members of the Inc Q incompatibility group have been exhaustively studied in an attempt to answer questions concerning the molecular basis of the broad-host-range capability. RSF1010 in particular has had its biochemical and genetic functions well characterized.

i. Plasmid RSF1010 Structure and Replication

RSF1010 is a naturally occurring, 8,684 kb nonconjugative plasmid with a copy number of approximately 12/bacterial chromosome (Guerry, et al., 1974). The plasmid was originally isolated from *Salmonella panama* and is capable of broad-host-range replication in members of the *Enterobacteriaceae*, but not in the *Pasteurellaceae*.

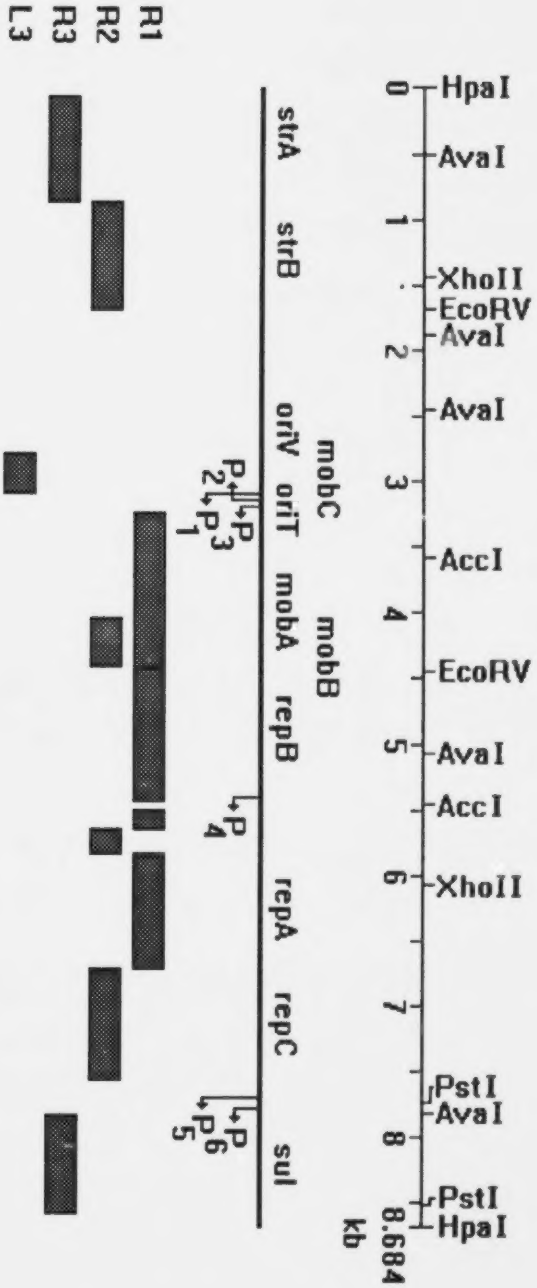
The whole of RSF1010 has been sequenced (Scholz, et al., 1989), and all of the gene products are in the process of being characterized. RSF1010 contains the sulfonamide II and the streptomycin A and B genes, responsible for resistance to these drugs in bacteria carrying the plasmid. The replication machinery has been well characterized, and the mobilization genes have been mapped out. RSF1010 lacks the transfer genes necessary for self-conjugative activity, but is fully mobilizable (Willetts & Crowther, 1981). With the completion of the DNA sequence, three previously unrecognized protein coding genes have been revealed (Scholz, et al., 1989). The major transcription signals for *E. coli* polymerase have also been identified (Scholz, et al., 1989). The organization of the RSF1010 genome is shown in Figure 1.

Replication of RSF1010 was characterized through the cloning, expression and purification of gene products, *in vitro* construction of the replication process, and *in vivo* and *in vitro* complementation studies of deletion mutants.

Figure 1. Physical map of RSF1010.

Distances (in kb) are shown on the top line, as are restriction endonuclease sites (from Scholz, et al., 1989). The approximate positions of the genes are indicated on the second line, as are the locations of the six known *E. coli* RNA promoters (arrows labeled *P1* through *P6*). The coding regions for the eleven identified RSF1010 proteins (shaded boxes) are aligned with the physical and genetic maps as well as assigned to their respective rightward (R1-R3) and leftward (L3) reading frames.

Figure 1. Physical map of RSF1010.


HpaI
AvaI
XhoII
EcoRV
AvaI
AvaI
AccI
EcoRV
AvaI
AccI
XhoII
PstI
AvaI
PstI
HpaI
0
1
2
3
4
5
6
7
8
8.684
kb
strA
strB
oriV
mobC
oriT
mobA
mobB
repB
repA
repC
sul
P1
P2
P3
P4
P5
P6
R1
R2
R3
L3

Analysis of RSF1010 DNA allowed the mapping of the active origin to an area of approximately 400 bp (DeGraff, et al., 1978; Scherzinger & Bagdasarian, 1984; Haring & Scherzinger, 1989), and showed that replication proceeded bidirectionally, using *rep* gene products (supplied in *trans*) encoded by a 4.7 kb region downstream of the replication origin (Scherzinger, et al., 1984; 1991; Haring, et al., 1985).

RSF1010 replication is independent of the functions of the *E. coli* gene products of *dnaA*, *-B*, *-C*, *-G*, *-I*, and *rpoB* (DeGraff, et al., 1978; Frey & Bagdasarian, 1989; Haring & Scherzinger, 1989; Scherzinger, et al., 1984; Honda, et al., 1991). Instead, three *rep* gene products, RepA, RepB (and its larger active partner RepB'), and RepC, function as specific DNA helicase, primase, and initiator protein, respectively, and are essential for plasmid replication (Haring & Scherzinger, 1989; Scherzinger, et al., 1984; Honda, et al., 1989). The RepB protein is a unique DNA primase in that it does not require ribonucleotide triphosphates for the priming reaction (Haring & Scherzinger, 1989), and it requires specific sites, *ssiA* and *ssiB*, located on each of the complementary DNA strands within the *oriV*, as the templates for DNA initiation (Scholz, et al., 1989; Haring & Scherzinger, 1989; Honda, et al., 1988.). The only known host factors required for replication of RSF1010 are DNA polymerase III (Frey & Bagdasarian, 1989; Scherzinger, et al., 1991), DNA gyrase (Scherzinger, et al., 1991), and single-stranded DNA binding protein, or SSB (Scherzinger, et al., 1991).

The *rep* proteins of RSF1010 bind in specific areas of the *oriV* to effect the initiation of replication: RepC binds to the *inc* repeats of the replication origin, which may catalyze the melting of an A-T rich region immediately downstream of the direct repeats. RepA helicases bind (presumably) to the A-T rich region and unwind the duplex to expose the *ssi* sites. Host SSB stabilizes the single-stranded DNA. RepB primase forms the priming complexes with the *ssi* sites, and host polymerase III holoenzyme extends the synthesis reaction bidirectionally (Scherzinger, et al., 1991; Haring & Scherzinger, 1989).

Plasmids are maintained in bacterial cells at a constant number which is characteristic for each individual or group of replicons. This is a result of a functional balance between positive regulation, mediated by initiation factors, and negative regulatory elements. The effect of incompatibility between two plasmids is a result of identity or close similarity in the negative regulatory mechanisms of the two replication systems. Negative regulation is important in plasmid replication, as no naturally-occurring replication systems can function properly without adequate negative control (Frey & Bagdasarian, 1989).

The IncQ origin has several regulatory sites. The 20 bp direct repeats (*inc* repeats) are essential for origin function (Scherzinger, et al., 1991; Haring & Scherzinger, 1989; Frey

& Bagdasarian, 1989; Persson & Nordström, 1986), and the sequential loss of the *inc* repeats cloned into a plasmid vector results in a concurrent decrease in the expression of incompatibility with an IncQ plasmid (Persson & Nordström, 1986; Frey & Bagdasarian, 1989). This can be explained by a titration of the RepC replication initiator protein that binds specifically to these repeats, and suggests that the RepC protein concentration in the cell is limiting and positively regulates the frequency of RSF1010 replication (Haring, et al., 1985; Frey & Bagdasarian, 1989). The replication genes themselves are under feedback regulatory control. The *rep* genes are expressed as two operons, a long one, initiated at the promoters P1/P3 and extending through the *repBB'AC* operon (see Figure 1), and a short one, initiated at the promoter P4 and extending through the *repA/C* cluster (Frey & Bagdasarian, 1989; Frey, et al., 1992; Maeser, er al., 1990). Through studies of deletion derivatives, the promoter cluster P1/P2/P3 was found to be repressed by high levels of MobC and RepB, which presumably attach to this region, possibly at the *oriT* site (Frey, et al., 1992). The F gene of RSF1010 (designated *cac*, for control of A and C genes), located at the beginning of the *repA/C* cluster and transcribed from both P1/P3 and P4, was found to encode a protein which attaches specifically to the region of the P4 promoter, and negatively regulates the expression of the *repA/C* operon (Maeser, er al., 1990; Frey, et al., 1992).

The interaction of several genes is required for the replication of IncQ plasmids. In RSF1010, the autoregulated *rep* genes provide plasmid specific functions of initiator protein, helicase and primase. RSF1010 therefore encodes its own proteins essential for primosome assembly and copy number control. This property is believed to render plasmid replication independent of host replication functions and to allow its propagation in a wide variety of bacterial hosts.

ii. pHD8.1 and pHD148 Plasmid Systems

For decades, sulfonamides have been used to successfully treat the sexually transmitted disease chancroid, the causative agent of which is the gram-negative bacterium *Haemophilus ducreyi*, a member of the *Pasteurellaceae* (Morse, 1989). Sulfonamide resistance in *H. ducreyi* is now seen throughout the world (Bilgeri, et al., 1982; Sturm, 1987; Taylor, et al., 1985).

Since sulfonamide resistance is known to be plasmid mediated in the *Enterobacteriaceae*, the possibility that sulfonamide resistance in *H. ducreyi* is also plasmid borne has been examined, along with the possibility that such *H. ducreyi* plasmids could be related to those found in the *Enterobacteriaceae* (Albritton, et al., 1982).

In the study conducted by Albritton, et al., 1982, all sulfonamide resistant strains of *H. ducreyi* were found to contain a 7.9 kb plasmid that conferred resistance to sulfonamide, but not streptomycin, and was easily transformed into *E. coli* C600. Filter-blot hybridization demonstrated that this plasmid, designated pHD148, was homologous to the linked sulfonamide-streptomycin resistance plasmid RSF1010 (Albritton, et al., 1982). EM heteroduplex analysis was used to demonstrate the relationship between pHD148 and RSF1010. TnA insertion mutants of RSF1010 (Heffron, et al., 1975) in the sulfonamide gene of RSF1010 (Ap101) and the streptomycin gene (Ap111) were used to facilitate identification of non-homologous regions between the two plasmids (Albritton, et al., 1982). The results were surprising in that 79% of the pHD148 and 69% of the RSF1010 sequences were found to be homologous, demonstrating an extensive similarity between the two plasmids (Albritton, et al., 1982).

Resistance to antibiotics within other members of the *Pasteurellaceae* has also been noted recently. *Actinobacillus pleuropneumoniae*, the causative agent of porcine pleuropneumonia, is of considerable economic importance causing chronic disease and death in the hog industry and has acquired some resistance to the tetracycline, penicillin, and streptomycin combinations used to treat the disease (Willson, et al., 1989b). The presence of an 8.1 kb, linked sulfonamide-streptomycin resistance plasmid in *A.*

pleuropneumoniae (called pHD8.1) related to pHD148 has been described recently (Willson, et al., 1989b).

The host range of pHD8.1 was found to mimic that of pHD148 in that the plasmid was capable of expressing stable resistance to streptomycin and sulfonamide in *E. coli* and *H. influenzae*, as well as in its native host, *A. pleuropneumoniae* (Willson, et al., 1989b). Restriction endonuclease digestion fragment sizes of pHD8.1, pHD148 and RSF1010 were similar after digestion with certain enzymes, but were strikingly different after digestion with others (Willson, et al., 1989b). Southern blotting using probes derived from restriction fragments of the three plasmids suggested that all three shared extensive regions of homology, along with unique regions of DNA sequence (Willson, et al., 1989b).

Since the publication of the paper by Willson, et al., 1989b, some of the areas of pHD148 and pHD8.1 have been sequenced in an attempt to understand how pHD148 and pHD8.1 can replicate within the *Pasteurellaceae*, while RSF1010 cannot, given the homologous nature of the three plasmids. It was found that the regions corresponding to the RSF1010 *oriV* have been deleted from both pHD148 and pHD8.1 (Albritton, Dixon & Willson, unpublished results). Since genes homologous to the *rep* genes of RSF1010 are present and the plasmids are known to replicate as broad-host-range (Willson, et al., 1989b), the idea that the replication mechanism was possibly other than that of IncQ plasmids is incorrect. The absence of the *oriV* region in both pHD8.1 and pHD148 raises the question of whether or not this region interferes with replication of these plasmids in the *Pasteurellaceae*. The 5' end of the pHD148 and pHD8.1 *oriV* region deletions occur in noncoding sequences (downstrean of the G gene and downstream of the I gene, respectively). Both the H and I genes, responsible for streptomycin resistance, are absent in pHD148 (Albritton, Dixon & Willson, unpublished results).

Sequencing data also revealed that the *oriT* region of RSF1010 is present in both pHD148 and pHD8.1, but that there are several deletions in that region common to both plasmids, suggesting that pHD148 and pHD8.1 are more related to each other than either are to RSF1010 (Albritton, Dixon & Willson, unpublished results).

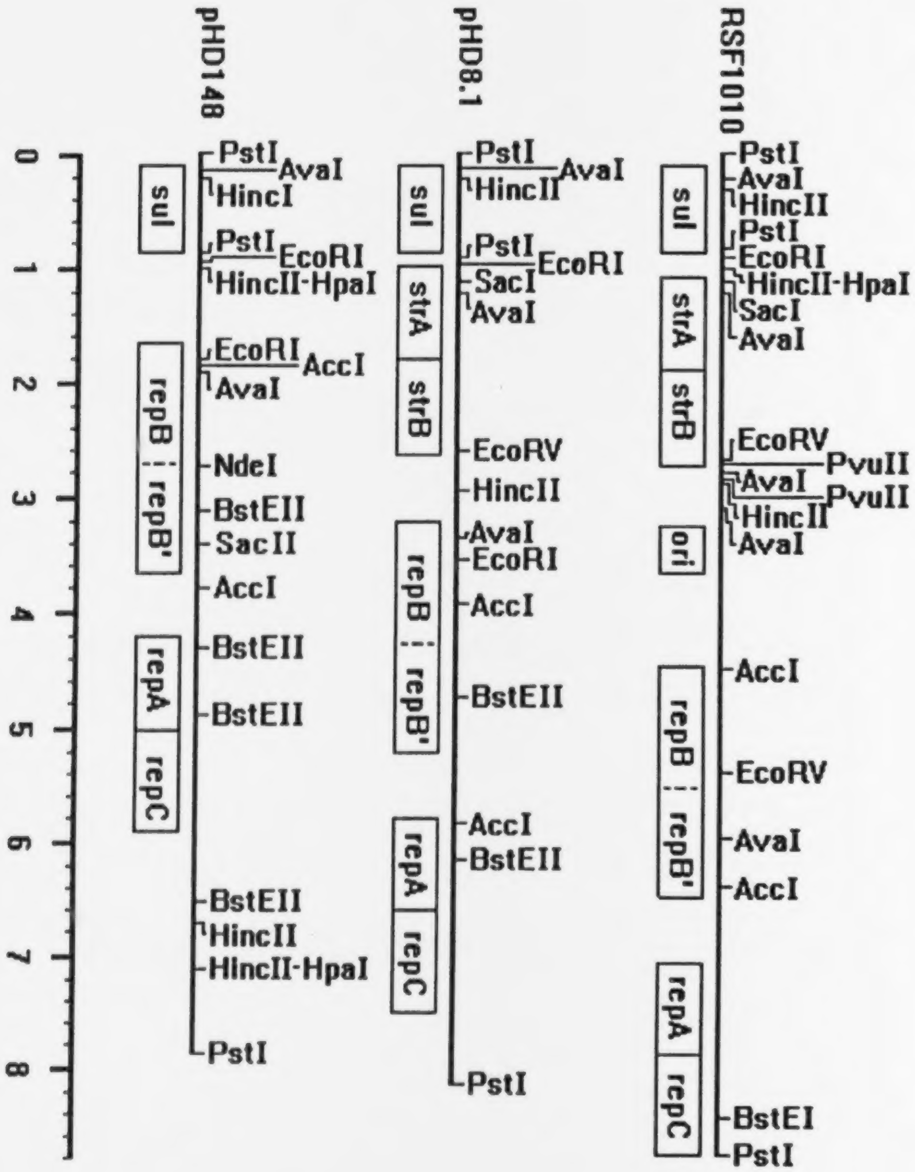
In pHD148, the 150 bp region immediately downstream of the G gene has no homology to either pHD8.1 or RSF1010, but does have homology to a region of pGS05 (a 100 kb selftransmissible plasmid, not from the IncQ incompatibility group) which flanks the sulfonamide resistance gene (Rädström, et al., 1991; Albritton, Dixon & Willson, unpublished results). The DNA sequence immediately upstream of the G gene of pHD148 also has homology to the corresponding region of pGS05 (Rädström, et al., 1991; Albritton, Dixon, & Willson, unpublished results). This suggests that the *sul*II region of pHD148 is more closely related to that of pGS05 than RSF1010. The physical maps of pHD148 and pHD8.1 are shown in Figure 2.

Figure 2. Restriction maps of plasmids RSF1010, pHD8.1, and pHD148.

The map at the top represents the entire RSF1010 plasmid linearized at the *Pst*I site immediately 5' to the sulfonamide resistance gene. The locations of restriction endonuclease sites and genes were determined by Scholz, et al., 1989. The size from 0 to 8.8 kb is indicated by the scale at the bottom.

The maps at the center and bottom represent the plasmids pHD8.1 and pHD148, linearized at the *Pst*I site immediately 5' to the sulfonamide resistance gene. The location of restriction endonuclease sites were determined by Willson, 1989. The presence and positions of the genes are approximate, and are based on DNA-DNA hybridizations done by Willson, 1989.

Figure 2. Restriction maps of plasmids RSF1010, pHD8.1 and pHD148.


pHD148
pHD8.1
RSF1010
0
1
2
3
4
5
6
7
8
sul
strA
strB
repB
repB'
repA
repC
ori
PstI
AvaI
HincI
HincII
EcoRI
HincII-HpaI
AccI
NdeI
BstEII
SacII
SacI
EcoRV
PvuII
BstEI

3. OTHER NATURALLY OCCURRING PLASMID VECTORS

i. The Plasmid pLS88

A 4.8 kb plasmid from a clinical isolate of *H. ducreyi* conferring resistance to sulfonamides, streptomycin, and kanamycin was isolated in a 1982-1983 study in Thailand (Taylor, et al., 1985) and recently characterized (Willson, et al., 1989a). This broad-host-range plasmid, pLS88, was shown through Southern blotting to have significant homology to the RSF1010 sulfonamide-streptomycin genes, and also to the kanamycin resistance transposon, Tn*903* (Willson, et al., 1989a). pLS88 was shown to be from an incompatibility group other than RSF1010 or pDM2 (Willson, et al., 1989a), and is capable of replication in members of the *Enterobacteriaceae* (Willson, et al., 1989a).

It is unlikely that pLS88 replicates by the same mechanism as RSF1010, as the essential *repA*, *repB*, *repC* region of RSF1010 is absent from pLS88 (Willson, et al., 1989a). Since approximately 3.0 of the 4.8 kb of the plasmid is occupied by antibiotic resistance determinants, pLS88 appears to contain a origin of replication that is small in size (thus resembling the structure of narrow-host-range plasmids) and may be more like RK2, which also has a small-sized replicative origin similar to narrow-host-range plasmids, but replicates as a broad-host-range plasmid (Kues & Stahl, 1989; Thomas & Helinski, 1989). The restriction maps of pLS88 and the deletion derivative pPC100 are shown in Figure 3.

4. RECOMBINANT PLASMID VECTORS

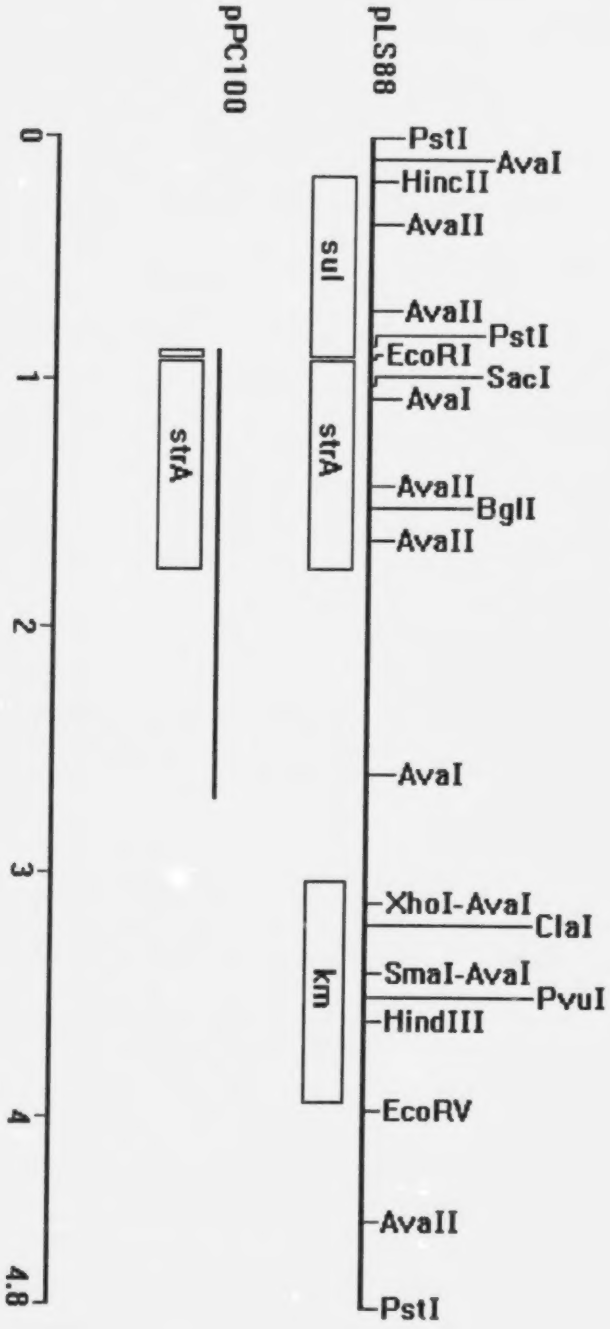
i. Known Vector Systems

There are no commercially available vectors capable of replication in the *Pasteurellaceae.* Recently, a number of researchers have attempted to create, through cloning, vector systems useful in both the *Pasteurellaceae* and in other bacterial families. Plasmids pHCV5, pHVT1 (Danner & Pifer, 1982) and pDM2 (McCarthy, et al., 1982) constructed from a naturally occurring *H. influenzae* plasmid will replicate within *H. influenzae* and *E. coli*, but are poorly transformable in *Haemophilus* sp. due to the lack of an "uptake site", a specific 11 bp sequence which directs preferential uptake of DNA fragments by competent cells during genetic transformation (Danner, et al., 1980). Plasmids pYG53 and pYG54 (a mobilizable derivative of pYG53) were constructed using a naturally occurring plasmid from *A. pleuropneumoniae*, called

Figure 3. Restriction maps of plasmids pLS88 and pPC100.

Distances from 0 to 4.8 kb are shown on the top line, as well as restriction endonuclease sites determined by Willson, 1989. The map in the center represents the entire pLS88 plasmid linearized at the *Pst*I site immediatly 5' to the sulfonamide resistance gene. The bottom map represents the pLS88 deletion derivative pPC100, lined up to correspond with the pLS88 map. The approximate positions of the pLS88 and pPC100 antibiotic resistance genes (open boxes) were determined by Willson, 1989.

Figure 3. Restriction maps of plasmids pLS88 and pPC100.


pPC100
pLS88
0
1
2
3
4
4.8
sul
strA
strA
km
PstI
AvaI
HincII
AvaII
AvaII
PstI
EcoRI
SacI
AvaI
AvaII
BglI
AvaII
AvaI
XhoI-AvaI
ClaI
SmaI-AvaI
PvuI
HindIII
EcoRV
AvaII
PstI

pYG10, which is capable of replication in both *E. coli* and *S. typhimurium* (Lalonde & O'Hanley, 1989; Lalonde, et al., 1989). Plasmids pJFF224-NX and pJFF224-XN are based on a minimum replicon of RSF1010, chloramphenicol resistance gene, and a gene expression cassette based on the *E. coli* bacteriophage T_4 gene 32 promoter region and transcription stop signal, separated by a segment of multiple cloning sites in both orientations. These vectors can replicate in *A. pleuropneumoniae*, *P. haemolytica* and *E. coli* (Frey, 1992). Plasmids pBAC504, pBAC509 and pBAC64 were created from the origin of replication of the 3.5 kb *P. multocida* plasmid pBAC1, pUC18, and antibiotic resistance genes from various sites. These plasmids can be introduced efficiently into both *P. multocida* and *E. coli* (Bills, et al., 1993).

ii. The plasmid pDM2

RSF0885 is a 5.7 kb *Haemophilus* ampicillin resistance plasmid (DeGraaf, et al., 1976). Several researchers have utilized the replication machinery of RSF0885 in the construction of new cloning vehicles for use in the *Pasteurellaceae*. One group used the replication origin and ampicillin resistance determinant of RSF0885, coupled with the pBR322 origin (to facilitate replication within *E. coli*), and tetracycline resistance determinant from either pBR322 (recombinant vector is pHCV5) or from the transposon Tn*10* (recombinant vector is pHVT1) for use in transfection of *H. influenzae* or *H. parainfluenzae* (Danner & Pifer, 1982). The second group used all of RSF0885 joined to a portion of p2265, a 55 kb *H. influenzae* conjugative plasmid encoding resistance to tetracycline and chloramphenicol (Albritton, et al., 1981; Albritton & Slaney, 1980), resulting in a 9.8 kb plasmid with two antibiotic resistance genes (McCarthy, et al., 1982). This plasmid, called pDM2, is capable of stable replication within both *H. influenzae* and *E. coli* (McCarthy et al., 1982). The restriction maps of RSF0885, p2265 and pDM2 are shown in Figure 4.

RSF0885 has been well studied, and the complete sequence is given in Figure 5 (Chen & Clowes, 1987; Heffron & McCarthy, 1979; Gilbride & Brunton, 1990; Albritton, et al., 1994; Albritton, unpublished results). Work done on resistance gene amplification in *H. influenzae* plasmids has resulted in a rough map of the tetracycline/chloramphenicol resistance region of p2265, and therefore of pDM2 (Spies, et al., 1983). Organization of the resistance genes and surrounding structures suggests the possibility of a chloramphenicol resistance transposon in *Haemophilus* sp., although amplification of the chloramphenicol resistance gene in these plasmids was not observed (Spies, et al., 1983).

Figure 4. Restriction map of plasmids RSF0885, p2265 and pDM2.

The map at the top represents the 4.0 kb *Bam*HI fragment of p2265 containing the chloramphenicol resistance gene ("Cm" box). The map in the middle represents the entire RSF0885 plasmid, linearized at one *Bam*HI site. The map at the bottom is pDM2, created from the insertion of the p2265 fragment into the second *Bam*HI site of RSF0885, upstream of the ampicillin resistance gene ("Am" box), as is shown. The locations of restriction endonuclease sites and antibiotic resistance determinants (open boxes) were determined by McCarthy, et al., 1982. The size from 0 to 10.0 kb is indicated by the scale at the bottom.

Figure 4. Restriction map of plasmids RSF0885, p2265 and pDM2.

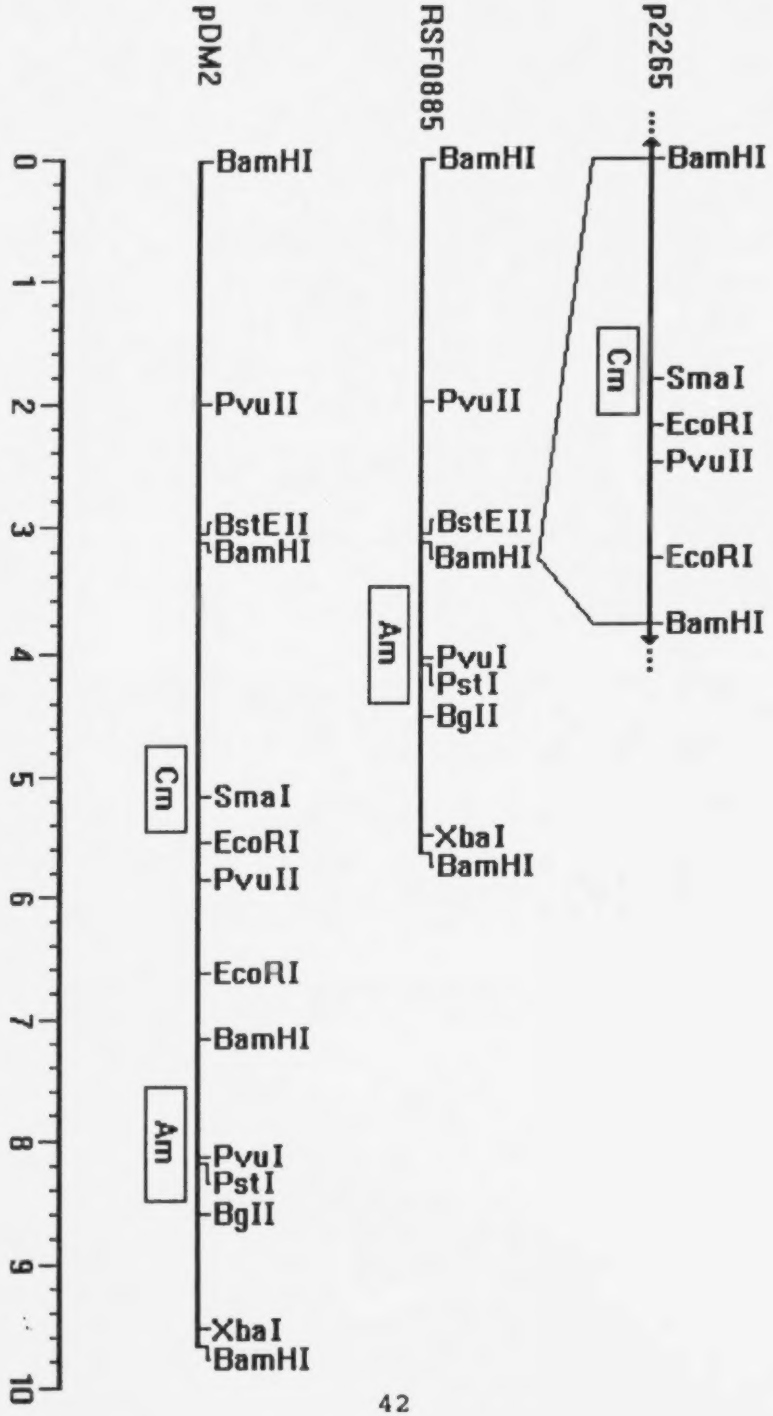

pDM2
RSF0885
p2265
0
1
2
3
4
5
6
7
8
9
10
BamHI
PvuII
BstEII
BamHI
Cm
SmaI
EcoRI
PvuII
EcoRI
BamHI
Am
PvuI
PstI
BglI
XbaI
BamHI
BamHI
PvuII
BstEII
BamHI
Am
PvuI
PstI
BglI
XbaI
BamHI
BamHI
Cm
SmaI
EcoRI
PvuII
EcoRI
BamHI

Figure 5. The composite nucleotide sequence of RSF0885.

Portions of the sequence were determined by several groups. Bases 1-485 and 4482-5599 were sequenced by Gilbride & Brunton, 1990. Bases 486-2906 were sequenced by Albritton & Noble (unpublished results). Bases 2906-4563 were sequenced by Heffron & McCarthy, 1979 and Chen & Clowes, 1987. The entire plasmid is 5599 bases in length.

The sequence as shown begins after the second *Bam*HI site (downstream of the ampicillin resistance gene, as seen in Figure 4). The arrows seen from position 106-279 represent repeat sequences of the plasmid origin. The double dashed line following the repeat sequences represent an AT rich region. The 39 K protein sequence, bracketed by a dashed line, begins at position 104 and proceeds leftward to position 4727, and is necessary for plasmid replication (Gilbride & Brunton, 1990).

The sequence between positions 2906-4562 consists of the 3' end of the transposon Tn*2*, beginning in the middle of the transposon's tnpR gene, encoding a regulator protein, through the ampicillin resistance gene (*bla*), both bracketed by a dashed line, and ending at the left hand inverted repeat of the transposon, indicated by a double dashed arrow (Heffron & McCarthy, 1979).

The *Bam*HI site into which the 4.1 kb p2265 fragment was inserted is indicated by a solid arrow.

Figure 5. The complete nucleotide sequence of RSF0885.

```
RSF0885           ATAGTCCCAATAGTTAAAGCTAAAATTCTGATTTCATCAATACTCAATCGGTAATTGGCT
                  <--------------- <-- 39K protein, 5' end <-- -------------

     61   TCCATAAGGCTATTAGCCTTTACAACAACTAAATCATTTGGCATAAGACAACAAATTTCCTGTTTAAAAC
          -------------------------------------------| ------------------>

    131   AACAAGCAAAATATACCTGTTGTTTATATATAAAACAACAAGTATTTTCTTAAAAGTTGTCTATAACAGG
                                                                    <---------

    201   AAATTTGTTGTCTTATAACAGGAAATTTGTTGTCGTATAACAGGAAATTTGTTGTCGTATAACAGGAAAT
          -----------<---------------------<---------------------<------------

    271   TTGTTGTCGTATAAGTTTGTAACTTATTGATTTTACTGGTTTTAAAAACGCCGAAAACAAGTAAAAAACA
          -------                                 |=============================

    341   AAAATATAAAAATATAGGGACTTTCCCGTCTTTTTTGGGGCTTTCAGCCCTAATTTTTTCTTTTTTTCAG
          ======================================================================

    411   GATTTAAAATTACAAAACCCTTACAGAGCAAGTAAACTTGTTTGCTTGTTCTGCAAGGGTTCAGCAACCG
          ================|

    481   TAGCCGTCAGGCGTAGGGCGGTAGCCTATAAAAGCCATTTAATTTTATCTTTAAACTTCCTTTTAAATGC
    551   TTTGAGTGGGTGTCTTTTATCGTACTCATCAATCCTTTTTTGCATTCTTTCGTTTGCTTTGTGATCGGCA
    621   AATTTTGAATAAGATTTTTCCATCTCATCTAACATTCTATCAATCCGTTTTTTATGTTGCCATTTCAGGT
    691   AAACATAAACACTTATAGCAATTAAAGACAATATCAATACATTGTAAAAAATGATTGTTACAATTTCGCT
    761   CACAGTTATTTTTTACCTTTTTCAATTTCTTCATTGATAAATGCACTCAATTCATCAAATTTCTTGTCAT
    831   CATTGATAAATTTACGCAACTTAGGGAAGTTTCTATCTACATCTAAAAGAGGGTTATTTATTATTTCATT
    901   TAGCCAAAAAGCCCCTAATAAAACCTTGTAATGCGTAGCTTTCTTACGCTTTTCTGCTTGTTCTTTTGAC
    971   TTAATCGCACGAATTTTCGCTTTGATTTCGTCCTGCTTGCGTTGTAAATCTGCTTGTTGCTGTTCCAATC
   1041   TTGTAAGTTTTTCGCTTGCCATACTAGCCCCTTTATATAGTTAGAAATTATCGTTATTTTATTCAGTAGG
   1111   TGCTAGGCTTGCAAGTGTTCTGTTCATTACGTTAAAATAACGTAATGCCCACTTATCAGTTTCTCTTCGA
   1181   GAAACTGGTGGGCAAGCGTACCGCTTGACCGTTTCGCAATACTCAACACTATGGCAATCTATCATTTAAA
   1251   CGTTCGCTATTGCAGTAAAAGCAAAGGGCAATCAGCTCAAGCCAAAAACGACTACATCAACCGCAATGAT
   1321   AAATATTCAAAGCGGTTAGATGATTTACAGTTTTCAGGCTATGGTAATATGCCAAAATTTGCCGAAGATA
   1391   ATCCGCAAGAATTTTGGCGATTGTCAGATATTTACGAGCGAGCTAATGCCCGAGTTTGTACTGAAATTGA
   1461   ATTTGCTTTACCTAGAGAATTAACCCTAGAACAACAGCAAAAATTAGTAAGTTCGTTTATAGAAAATACG
   1531   GTTGATAGCGGTAGCAATAAAGTACCCTACTCTTTCGCTATCCATACCGATAAAAATAATCATAATCCCC
   1601   ATTGTCATTTGATATTTTCAGAACGCCAACTTGACGGCATAGACCGTACAGCCGAGCAGTTTTTTAAACG
   1671   TGCTAATACTAAATCCCCAGAAAAGGGCGGAGCGATGAAAACGGCAGATTTTCGAGATCGTGAGTTTATC
   1741   CAATCTGTCCGAAAAACGTGGAGAGAGCAAGCTAATCAAGCCTTAGAGCAATACGGATATGCCGCACGAA
   1811   TTGACGAACGTAGCTACAAGGAACAAGGCATAGAGCAAGCCCCAAGAGCAAGAATTGACAGGGTAACGTG
   1881   GCAAGAATTGAACCGATTAGAGCAAGAAGAACGCCAAATCGTGCAAGAGCTTGCACTTAAAGGACAAGAA
   1951   ATTAACAAAGAAAAATCCTACTTGCAGAAAATCGAAGAAAAACAGGCTCAAGGAATGGGCAAATATGAAT
   2021   CCAAATTCGCAGCTGCGTTTTCTAAATTATCGGAAAGTGCCCTAAAACACGATTTAAGCAACGAAAAAGA
```

```
                .         .         .         .         .         .         .
2091   AAAAGACAGTAAAATACACACTCAAGAAGAAAAAGTGCCTCAAAATCGCATTCAGGGGCTTTCTCAAGCA
                .         .         .         .         .         .         .
2161   GATTTTGATCAGTTTTTAATTGATGAATGGCTACCTCAAATAGAAAAATACGTTAAAGCCCAAGAAAAGC
                .         .         .         .         .         .         .
2231   GGGACGGAATGGAAGTAGAGATCACGCAATACGACAAGGATTTACAGCGTATTCAGGGAGACTATAACAA
                .         .         .         .         .         .         .
2301   GCTCACAGATAAAAATCAGGGTTTTCTCGGTTTATGGGAAACTAAAGAGCAAAAAGCAAAGAAAAAAGAG
                .         .         .         .         .         .         .
2371   CTTGAAGATGAATACAAACATACAGCAGAGCAACGGAACGCTAAAAGCCAAGAATTAGCCGAGTATAGCC
                .         .         .         .         .         .         .
2441   AAAAAATAAAAGCATACGAACAGAAAACGCTAGAGCCAATCAACGAGAAGATTGCCAAATATCAAGCTGA
                .         .         .         .         .         .         .
2511   CAACCCTGAAATAAAAATGCGGAGCTTAGGATTTGTGAAAAAAATTAAGGCTCAAGGGGCATATAAAGCG
                .         .         .         .         .         .         .
2581   GCTCAAGAGCGAATGGAGCGAGAAAAACAGCACCAACAGGAAAAACAACAGAGACATTTAGAGCGAGAGA
                .         .         .         .         .         .         .
2651   GTGGTTTGAGCTTGTAGCTAACGCCCTACGCCTACGGCTTCGGTTGTTCAACCCTTAAAGAACTCGCAAC
                .         .         .         .         .         .         .
2721   AAGTTGCAAATTCTTTAAGGGTTCGCAATAAAAACAACCCCTAAACATTTCTGCCCAGCGGTTGAAAATT
                .         .         .         .         .         .         .
2791   TACCTATTCACCATTACAATGATCAAGCAGGAAATTTTTTTGATTGCCGTAAATGTCCGTATATCTAGTT
                .         .         .         .         .         .         .
2861   GAGGCACAACCCGCCAAAGTCATTGCCCCAACCAGAACGGCGATAAACCGTATATTTACCGATAAGGCAT
                                                    |------------------------

                .         .         .         .         .         .         .
2931   CCGGCAGTTCAACAGACCGGGAAGGGCTGGATTTGCTGAGGATGAAGGTGGAGGAAGGTGATGTCATTCT
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3001   GGTTAAGAAGCTCGACCGTCTTGGCCGCGACACTGCCGATATGATCCAACTGATAAAGGAATTTGACGCT
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3071   CAGGGCGTGGCAGTCCGGTTCATTGATGACGGGATCAGTACCGACGGTGATATGGGGCAAATGGTGGTCA
       --------------------- --> tnp R, incomplete --> ----------------------

                .         .         .         .         .         .         .
3141   CCATCCTGTCGGCTGTGGCACAGGCTGAACGCCGGAGGATCCTAGAGCGCACGAATGAGGGCCGACAGGA
       ----------------------------------------------------------------------

                                           ↑ BamHI insertion site
                .         .         .         .         .         .         .
3211   AGCAAAGCTGAAAGGAATCAAATTTGGCCGCAGGCGTACCGTGGACAGGAACGTCGTGCTGACGCTTCAT
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3281   CAGAAGAGCACTGGTGCAACGGAAATTGCTCATCAGCTCAGTATTGCCCGCTCCACGGTTTATAAAATTC
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3351   TTGAAGACGAAAGGGCCTCGTGATACGCTTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAG
       ----------------------|

                .         .         .         .         .         .         .
3421   ACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAA
                .         .         .         .         .         .         .
3491   ATATGTATCCGCTCATGAGACAATAACCCTGGTAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAG
                                                                        |----

                .         .         .         .         .         .         .
3561   TATTCAACATTTTCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCA
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3631   GAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATC
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3701   TCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGT
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3771   TCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTAT
       ---------------------------- --> bla --> -----------------------------

                .         .         .         .         .         .         .
3841   TCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAG
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3911   AATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCTGCCAACTTACTTCTGACAACGATCGGAGG
       ----------------------------------------------------------------------

                .         .         .         .         .         .         .
3981   ACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCG
       ----------------------------------------------------------------------
```

```
               .         .         .         .         .         .         .
4051  GAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4121  GCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGA
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4191  TAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4261  GGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTA
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4331  TCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4401  GATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTT
      ---------------|
               .         .         .         .         .         .         .
4471  TAATTTAAAAGGACTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTC
                 ===========================================================
               .         .         .         .         .         .         .
4541  GTTCCACTGAGCGTCAGACCCCTATCTATAAACTCTTGGCTTGGTTCTAATCCCTCTAAACGATTATTAT
      =====================>
               .         .         .         .         .         .         .
4611  CAATAGCCGCTCTAACCGCTTTTTCTCGGCTTAATTTTTCTGTCTCTGTTATAAAATTGCTATTCATCTT
               .         .         .         .         .         .         .
4681  GTTCTTCTTCAAAAAAAAGTTAAGTAAAATACCTACCTAAATTTTTACTAGTTCGCAATCTACGAGCTTA
                                                   |------------------------
               .         .         .         .         .         .         .
4751  TAACCTCGTTTTTTCAATTCATTTAAAAAATCAGATTTTGAGCCTAATTTTGATCTATTGCTATCGTTAC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4821  CCGCTAGAAATACCCAGTAATTACGCAAATCTTCATTGGTAACTTTCGTAATATCGGTGTAATGATCTTC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4891  GAGTATTTTTAAGCAATCTCTAGCCCATAAACCGTACTCGTGATTGCTCATCTTAGGGTTTTGCTTATCG
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
4961  AGTTTGACGAACTTCCCATACTTGTTTTTATGTGGAAATACTGGCCGTTTTGCAACTTCTTCAATTTTTT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
5031  GAGCTGTTCGTTTTTTACTACCAATCACAAAATTTAAAGAGTGAATAGTACGCCCACGCTTGATTTGTTC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
5101  AACCTCAACGACTAAATCAGATTTCTCGTTAATCTCAGTTATTGCAGGTTCCAAAACACGTTGATTTAAT
      ------------------ <-- 39K protein, 3' end <-- -----------------------
               .         .         .         .         .         .         .
5171  GAATTAAATCTAGGGTATTTATTTTCAACCTGAAGCCATTCTTTTAGTTTTTCTACTGTAATTTCACGAC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
5241  TACCAACAGAGCGATATTGTGTAATTAGCTCATAAATTCGAATTGAATGTACACTGTTGAAATAAGAGAT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
5311  ATGTTTGAGTTGATATTGCGTGAATTGCCCTTTAAGTTGCGTTAGGTATGGCATAACTTCATCAGTCATT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
5381  GCAATTCTAAAACGCCCCTCTTTCTTGAAATATGTTCTAGAGGAAACCCAACGAAATTCAGTTACACGGT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
5451  CTTTATCTTCAGTTTTAACACTTCGGTCATAAATCCGTTTTATAGCCGCCTGAATTTGCTTATAGGCGTT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
5521  ATCTTGGCTTATTTCTGGAAACTCACGGACAAAATCAGCCACCGTAAAATCAAAAATCTTTTGATTACAT
      ----------------------------------------------------------------------

5591  TTCGGATCC
      <--------
```

iii. The Plasmids pPW87 and pPW88

The restriction endonuclease maps of the large conjugative IncW plasmid pSa, originally isolated from an epidemic strain of *Shigella* (Ireland Valentine, 1983; 1985; Tait, et al., 1982) indicate that *Pst*I digestion produces 3 fragments (33, 3.3 and 2.1 kb). However, other researchers have demonstrated the existence of an *E. coli* strain containing a heterologous population of pSa which produces 4 *Pst*I fragments, with a doublet at 3.2/3.5 kb (Willson, 1989). On solid media, the two forms segregate in single colony isolates, each containing homologous plasmid populations (Willson, 1989). The plasmid containing the larger fragment was designated pSa* (Willson, 1989).

The plasmids pPW87 and pPW88 were constructed by replacing the 0.8 kb *Pst*I fragment of pLS88 (containing the sulfonamide resistance gene) with the chloramphenicol resistance gene contained within the 3.2 kb (pPW87) or 3.5 kb (pPW88) *Pst*I fragment of pSa and pSa*, respectively (Willson, 1989; Willson, et al., 1989a). Restriction maps of pPW87 and pPW88 are shown in Figure 6. Interestingly, deletions occurred in both pPW87 and pPW88 when placed in *H. influenzae* or *A. pleuropneumoniae* and maintained in an environment containing specific antibiotics. When chloramphenicol resistance was selected for, no deletions occurred. However,

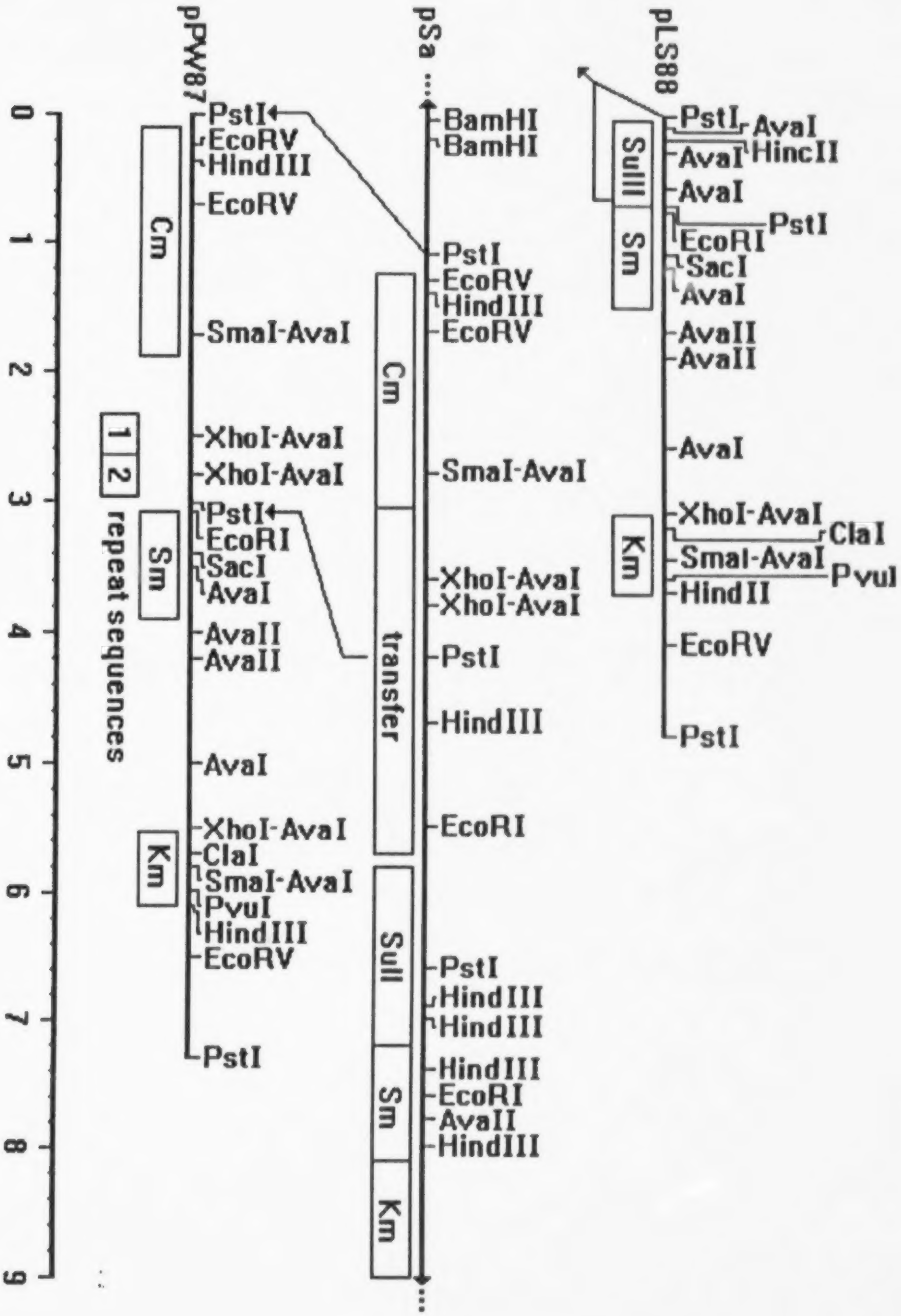
Figure 6. Restriction map of plasmids pLS88, pSa, pPW87 and pPW88.

The map at the top represents the entire pLS88 plasmid, linearized at the *Pst*I site immediately 5' to the sulfonamide resistance gene. The map in the center represents a part of the pSa plasmid containing most of the plasmid's restriction sites, antibiotic resistance determinants, and conjugal transfer region, and is based on work done by Ireland Valentine, 1985 and Tait, et al., 1982. The bottom map represents the entire of pPW87, created by the replacement of the 0.8 kb *Pst*I/*Pst*I fragment of pLS88 with the *Pst*I/*Pst*I fragment from pSa. The region of pSa within the *Pst*I/*Pst*I fragment which contains two long repeat sequences is indicated by the numbered boxes below the map.

The plasmid pPW88 was created as per pPW87, using the plasmid pSa*, which contains a third repeat sequence. This is seen on the pSa* and pPW88 restriction endonuclease maps as three *Xho*I-*Ava*I sites (not shown).

The size from 0 to 9.0 kb is indicated by the scale at the bottom.

Figure 6. Restriction map of plasmids pLS88, pSa, pPW87 and pPW88.


pPW87
PstI
EcoRV
HindIII
EcoRV
SmaI-AvaI
XhoI-AvaI
XhoI-AvaI
PstI
EcoRI
SacI
AvaI
AvaII
AvaII
AvaI
XhoI-AvaI
ClaI
SmaI-AvaI
PvuI
HindIII
EcoRV
PstI
Cm
Sm
Km
1
2
repeat sequences
0
1
2
3
4
5
6
7
8
9
pSa
BamHI
BamHI
PstI
EcoRV
HindIII
EcoRV
SmaI-AvaI
XhoI-AvaI
XhoI-AvaI
PstI
HindIII
EcoRI
PstI
HindIII
HindIII
HindIII
EcoRI
AvaII
HindIII
Cm
transfer
SulI
Sm
Km
pLS88
PstI
AvaI
AvaI
HincII
AvaI
PstI
EcoRI
SacI
AvaI
AvaII
AvaII
AvaI
XhoI-AvaI
ClaI
SmaI-AvaI
PvuI
HindII
EcoRV
PstI
SulII
Sm
Km

when streptomycin resistance was selected for, the chloramphenicol resistance gene was deleted; when kanamycin resistance was selected for, the chloramphenicol resistance gene was sometimes deleted (Willson, 1989). Recently, the chloramphenicol resistance gene of pSa was recognized to be within the In6 integron (Stokes, et al., 1993; Ireland Valentine, 1985); it is possible that the above deletions in pPW87 and pPW88 are as a result of integron structures cloned into pLS88 along with the chloramphenicol resistance gene.

II. THE PROBLEM: DEVELOPMENT OF VECTOR SYSTEMS FOR STUDY WITHIN THE *PASTEURELLACEAE*

There are no commercially available vector systems for genetic analysis of bacteria within the *Pasteurellaceae*. Because of the importance of these bacteria in diseases of both animals and humans, the broadening of our knowledge of pathogenic mechanisms, DNA transfer systems, and metabolic systems through genetic study is desireable.

The first step in the development of genetic tools for DNA and gene analysis is to find indigenous plasmids, hopefully containing one or more marker genes, and having a host range including bacteria outside the *Pasteurellaceae*. The plasmids pHD8.1, pHD148, and pLS88 have all of these characteristics. The recombinant plasmids pDM2 and pPW87/88 have been altered to conform to these characteristics.

The next step is to increase our knowledge of the genetic makeup of these plasmids. The gene organization should be understood, delineating essential from nonessential genes and DNA structures. Promoter regions, and replication regions and mechanisms should be mapped and studied. The presence and structure of special genetic elements, including transposons and integrons, should be investigated, as these can be useful additions to a vector system.

This study reports work undertaken to characterize several plasmid vectors and genetic elements that may be useful as tools for genetic analysis of members of the *Pasteurellaceae*.

III. MATERIALS AND METHODS

A. BACTERIA AND PLASMIDS

The plasmids and bacteria that were used in this study are listed in table 2.

Table 2. Bacterial strains and plasmids used in this study.

HOST	VECTOR	PHENOTYPE	SOURCE
ENTEROBACTERIACEAE			
Escherichia coli			
ATCC 33694	ND*	Su^{s}, Sm^{s}, Km^{s}	American Type Culture Collection
JM105	ND	Su^{s}, Sm^{s}, Km^{s}	Albritton, EC184
EC110	RSF1010	Su^{r}, Sm^{r}	Albritton, EC184
SM159	AP101	Sm^{r}, Ap^{r}	R. Gill
SM158	AP111	Su^{r}, Ap^{r}	R. Gill
EC111	pHD148	Su^{r}	Albritton, C600
EC186	pHD8.1	Su^{r}, Sm^{r}	Willson, AP37
EC196	pLS88	Su^{r}, Sm^{r}, Km^{r}	Slaney, *H. ducreyi* CH37
EC301	pPC100	Sm^{r}	Willson, HI18
EC253	RSF0885	Am^{r}	Willson, EC174

EC005	pDM2	Am^r, Cm^r	J.K. Setlow
EC180	pSa/pSa*	Cm^r, Sm^r, Km^r	Albritton, J53
EC198	pPW88	Cm^r, Km^r	Willson, EC196
Salmonella typhimurium			
LT2	ND	Su^s, Sm^s, Km^s	Salmonella Stock Culture
Proteus mirabilis			
UAH30	ND	Su^s, Sm^s, Km^s	clinical isolate
PASTEURELLACEAE			
Haemophilus influenzae			
HI14	ND	Nv^r, Sm^s	Albritton, Rd^{Nv}
SM59	p2265	Tc^r, Cm^r, Nv^r	Albritton, Rd^{Nv}
Actinobacillus lignierissii			
ATCC 19393	ND	Sm^s	American Type Culture Collection
Pasteurella multocida			
UAH40	ND	Sm^s	clinical isolate
PSEUDOMONACEAE			
Pseudomonas aeruginosa			
DGI	ND	Sm^s	clinical isolate
CAMPYLOBACTER			
Campylobacter jejuni			
UA580	ND	Sm^s	clinical isolate, D. Taylor
Helicobacter pylori			
UA877	ND	Sm^s	clinical isolate, D. Taylor
STREPTOCOCCACEAE			

Streptococcus pneumoniae			
UAH 50	ND	Sm^{s}	clinical isolate
Enterococcus faecalis			
ATCC 29212	ND	Sm^{s}	American Type Culture Collection
MICROCOCCACEAE			
Staphylococcus aureus			
ATCC 25923	ND	Sm^{s}	American Type Culture Collection

*ND - None Detected

B. MEDIA AND GROWTH CONDITIONS

Solid media used in this study consisted of 5% sheep's blood agar (Gibmar). For the cultivation of X- and V-factor requiring strains, chocolatized sheep's blood agar was used (Gibmar). *Proteus* sp. was grown on MacConkey agar (Gibmar) to prevent swarming. Liquid media for all uses consisted of BHI broth (Gibco).

Cultures were incubated at 35°C for 18 to 24 h. Members of the *Pasteurellaceae* were incubated in an atmosphere containing 5% CO_2. Cultures in liquid broth were agitated vigorously (100-150 rpm) during incubation.

C. ANTIBIOTICS

Antibiotics were incorporated into both liquid and solid media for the purposes of plasmid selection and plasmid maintenance. The concentrations of antibiotics that were used in the media are listed below:

	Solid Media	Liquid Media
Ampicillin	25 μg/ml	100 μg/ml
Chloramphenicol	15 μg/ml	50 μg/ml
Kanamycin	25 μg/ml	100 μg/ml
Streptomycin	25 μg/ml	100 μg/ml

D. ISOLATION OF BACTERIAL DNA

1. RAPID SMALL-SCALE PLASMID PREPARATION

5.0 ml of BHI broth supplemented with the appropriate antibiotic was inocculated with a single bacterial colony using a straight wire and incubated 16-18 hours at 35°C. As per the method of Takahashi and Nagano (Takahashi & Nagano, 1984), 1.5 ml of the turbid broth was transferred into an Eppendorf tube and centrifuged in a microfuge (Eppendorf 5414) for 5 minutes. The supernatant was aspirated and the cell pellet was suspended by vortexing in 200 µl of a solution containing 40 mM Tris-acetate, 2 mM EDTA, pH 8.0. 400 µl of a lysing solution containing 2% (w/v) SDS, 50 mM Tris stock, and 0.2 N NaOH was added, and the tubes were inverted 10 times, then incubated at room temperature for 5 minutes. 300 µl of a cold (4°C) neutralizing solution containing 3.0 M sodium acetate-acetic acid (pH 5.5) was then added, and the tubes were inverted 20 times, then incubated at 0°C for 5 minutes. The tubes were then centrifuged at room temperature for 5 minutes, stored at 0°C for 5 minutes, and centrifuged at 0°C for 10 minutes. The supernatant was decanted to a new tube and an equal volume of chloroform (approximately 700 µl) was added. The tubes were shaken briefly and centrifuged at 0°C for 3 minutes. 500 µl of the aqueous layer was transferred to a new tube and 1.0 ml of -20°C ethanol was added. The tubes were inverted 10 times and incubated at 0°C for 5 minutes, and then centrifuged for 5 minutes to collect the plasmid precipitate. The supernatant was removed and the pellet dissolved in a 25 µl solution containing 10 mM Tris-acetate and 2 mM EDTA, pH 8.0.

2. LARGE-SCALE PLASMID PREPARATION

A 1000 ml sterile flask (Pyrex) containing 500 ml of BHI broth with the appropriate antibiotic was inoculated with a single colony, and incubated in a shaking incubator at 35°C for 12 to 15 hours. As per the method by Sambrook, et al. (Sambrook, et al., 1989), the turbid broth was transferred into a centrifuge bottle and spun at 6 000 g for 5 minutes. The supernatant was removed and the cell pellet was suspended in 10 ml of a solution containing 50 mM glucose, 25 mM Tris-chloride, 10 mM EDTA, pH 8.0 by pipetting up and down vigorously. 1.0 ml of a 10 mg/ml solution of lysozyme in TE (pH 8.0), and 20 ml of a solution containing 0.2 N NaOH and 1% (w/v) SDS were added, and the contents of the bottles were mixed by gentle inversion. The bottles were stored at room temperature for 10 minutes. 15 ml of a 0°C solution containing 3.0 M potassium acetate in 11.5% (v/v) acetic acid was added, the contents of the bottles were mixed thoroughly by shaking, and incubated at 0°C for 10 minutes. Following incubation, the bacterial lysate was centrifuged at 3,500 g for 20 minutes at 4°C, allowing the rotor to stop without braking. The supernatant was filtered through 4 layers of cheesecloth into two 50 ml conical tubes, 0.6 volumes of isopropanol was added, and following vortexing, stored at room temperature for 10 minutes. The mixture was centrifuged at 3,500 g for 20 minutes at room temperature, the supernatant was removed, and the pellet was washed with 70% ethanol. After the pellet was allowed to dry, it was dissolved in 3.0

ml TE (pH 8.0). 3.0 ml of 0°C 5 M LiCl was added, the solution was mixed by vortexing, and centrifuged at 3,500 g for 20 minutes at 4°C. The supernatant was transferred to a fresh 50 ml conical tube and an equal volume of isopropanol was added. The solution was vortexed, and centrifuged at 3 500 g for 20 minutes at 4°C. The supernatant was removed, and the pellet was washed with 70% ethanol and allowed to dry. The pellet was dissolved in 500 μl of a solution containing TE (pH 8.0) with 20 μg/ml bovine pancreatic RNAse and incubated at room temperature for 30 minutes in an Eppendorf tube. 500 μl a 1.6 M NaCl, 13% (w/v) PEG 8000 solution was added. The mixture was vortexed, then centrifuged at 12,000 g for 5 minutes at 4°C. The supernatant was removed by aspiration and the pellet dissolved in 350 ml of TE (pH 8.0). The solution was extracted with equal volumes of phenol (pH 8.0), then phenol:chloroform (1:1), then chloroform. The aqueous phase was transferred to a new micgofuge tube and 150 μl of 10 M ammonium acetate was added. The solution was vortexed well, 1.0 ml of ethanol was added, and the mixture incubated at room temperature for 10 minutes. The DNA was then pelleted by centrifugation at 12,000 g for 5 minutes at 4°C. The supernatant was removed by aspiriation, washed with 70% ethanol, allowed to dry, then dissolved in 720 μl of sterile distilled deionized water.

The plasmid DNA was purified by CsCl-density gradient centrifugation as described by Garger, et al. (Garger, et al., 1983). The DNA solution was mixed with 1.26 g of CsCl, 120 μl of 10 mg/ml ethidium bromide was added, and the resultant solution was placed in a 2.2 ml polyallomar Quick Seal tube, layered beneath 1.6 ml of 65% (w/v) CsCl. The remaining tube volume was filled with mineral oil, and the tube was sealed. CsCl density gradients were formed by centrifugation in the Beckman table top TL-100 ultracentrifuge for at least 4 hours at 100,000 rpm, acceleration set at "5", and deceleration at "0". Banding patterns were observed using a Wood's lamp, and the plasmid-containing band (the lower band) was removed using a needle and syringe. The ethidium bromide was removed through extraction with water saturated n-butanol. The DNA-containing solution was transferred to a 50 ml conical tube. Two volumes of TE (pH 8.0) was added, and the solution was brought to 0.3 M sodium acetate using a 3.0 M stock (pH 5.5). One volume of isopropanol was added, and the mixture was frozen on dry ice. After thawing, the solution was centrifuged at 3,500 g for 1.5 hours. The supernatant was removed, the pellet washed with 70% ethanol, and allowed to dry. The dry pellet was dissolved in 150 μl of TE (pH 8.0).

E. AGAROSE GEL ELECTROPHORESIS

Agarose was used at 0.8%, unless otherwise specified, in 0.5 TBE buffer (5 x TBE is 0.45 M Tris base [54 g/l], 0.45 M boric acid [27.5g/l], and 0.01 M EDTA [20 ml of 0.5 M EDTA, pH 8.0]) with 100 μg/l ethidium bromide. The agarose suspension (0.8 g in 100 ml 0.5 x TBE) was boiled until the agarose dissolved, cooled to approximately 60°C, ethidium bromide was added, and poured into either a 10 x 15 cm or a 8 x 10 cm plastic gel-casting tray (Bio-Rad) containing a 1 mm thick comb. After the gel had set, the comb was removed and the gel was submerged in an appropriately sized "mini-sub" electrophoresis cell (Bio-Rad) containing 0.5 x TBE with ethidium bromide. Samples of DNA to be electrophoresed were combined with a 6 x gel loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol FF, and 40% (w/v) sucrose in water) and placed in the gel wells using a micropipette (Gilson) with a disposable tip. The electrophoresis system was run using a constant voltage (100 V, unless otherwise specified) for 1 to 1.5 hours. Bands of DNA were visualized by illumination with long-wave ultraviolet light. Photographs were taken by exposure to type 55 film gel, an orange celophane filter, and f = 4.5 for 20 to 30 seconds.

Bands with DNA to be used in further experiments were excised with a scalpel and placed in an Eppendorf tube with 3.0 volumes of NaI (6 M). After the agarose was dissolved (65°C for 5 minutes), 10 μl of "Glassmilk" (a silica matrix, Bio 101) was added. After 15 minutes at room temperature, the matrix and bound DNA was pelleted by centrifugation in a microfuge, washed 3 times with "New" wash (a solution of NaCl/Tris/ethanol/EDTA, Bio 101), and eluted 3 times in 10 µl of 0.1 x TE, pH 8.0. The eluates were combined, and spun to pellet residual silica matrix. The DNA was stored at - 20°C until used.

F. POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel electrophoresis (12% and 20%) was used to separate protein products of *in vitro* translation of pLS88, pPC100 and pPW88. A 1.5 mm spacer was clamped between 2 clean glass plates (18 x 16 cm) and assembled in a casting stand (Bio-Rad). As per the method of Sambrook, et al. (Sambrook, et al., 1989), the resolving gel solutions (30 ml) were composed of the following:

	12% gel	20% gel
water	9.9 ml	1.9 ml
29% (w/v) acrylamide/1% (w/v) N,N'-methylenebisacrylamide	12.0 ml	20.0 ml
1.5 M Tris (pH 8.8)	7.5 ml	7.5 ml
10% SDS	0.3 ml	0.3 ml
10% ammonium persulfate	0.3 ml	0.3 ml
TEMED	0.012 ml	0.012 ml

The solutions were poured between the plates, and allowed to set for 30 minutes. The stacking gel (5%, 10 ml) was composed of the following:

water	6.7 ml
29% (w/v) acrylamide/1% (w/v) N,N'-methylenebisacrylamide	1.7 ml
1.5 M Tris (pH 8.8)	1.25 ml
10% SDS	0.1 ml
10% ammonium persulfate	0.1 ml
TEMED	0.01 ml

The solution was poured between the plates, a comb was inserted, and polymerization was allowed to occur at room temperature. When polymerization was complete (about 45 minutes), the comb was removed and the wells washed with distilled water. The gel was placed in a vertical gel electrophoresis system (Bio-Rad 220) containing a Tris-glycine buffer (25 mM Tris, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS) and air bubbles adhering to the bottom of the gel were removed. Samples (usually 10-15 μl) diluted 1:1 with loading buffer (0.08 M Tris-HCl (pH 6.8), 0.1 M DTT, 2% (w/v) SDS, 10% (v/v) glycerol, 0.1 mg/ml bromphenol blue) were heated to 100°C for 5 minutes to denature the protein, and loaded into wells using a micropipette (Gilson) and disposable tip. An electric potential of 150 VDC was used to move the dye front into the stacking gel, and then a constant current of 28 mA was used to electrophorese the proteins through the resolving gel (3-4 hours). At the end of the electrophoresis, the current was turned off, the plates were separated, and the gel fixed in 5-10 volumes of 7% acetic acid for about 15 minutes (until the bromphenol blue had turned yellow). The gel was washed briefly in deionized water, placed flat on dry Whatman 3 mm filter paper, covered with Saran Wrap, and dried using a gel dryer (model SE 1160, Hoefer Scientific Instruments) at low heat (50-65°C). Visualization of the protein bands was achieved through autoradiography, detecting the L-[^{35}S] methionine label incorporated into the protein products during translation. Protein standards used were [^{14}C] methylated rainbow markers (mw 14.3-200kDa) from Amersham Inc. The dried gel was exposed to Kodak X-OMAT XAR-5 8" x 10" film at room temperature, and developed after 24 hours.

G. ENZYMATIC REACTIONS INVOLVING DNA

1. RESTRICTION ENDONUCLEASE DIGESTION

The enzymes used in this study were obtained from either New England Biolabs or Boehringer Mannheim Corp. Digestion buffers and temperatures were as recommended by the manufacturers.

2. LIGATION

Fragments of DNA to be ligated were separated by agarose gel electrophoresis and recovered by extraction from the gel as previously described. T4 DNA ligase was obtained from Boehringer Mannheim Corp., and the ligation reaction buffer was as recommended by the manufacturer. The reaction was allowed to proceed at 14°C for 20-24 hours.

3. DNA TRANSLATION

DNA transcription and translation of pLS88, pPC100, and pPW88 was done using the *E. coli In vitro* Prokaryotic Translation kit, following the recommendations of the manufacturer (Amersham), following the procedure for L-[^{35}S] methionine labeling of protein products.

H. TRANSFORMATION

DNA transformation of bacteria was done *via* electroporation (Bio-Rad Gene Pulser). Bacteria grown in broth or on solid media for 16 hours were suspended in electroporation buffer (EPB: 0.3 M sucrose, 0.3 mM Na_2HPO_4, 0.2 mM KH_2PO_4, 19% glycerol), and washed in EPB 3 times. Following the last wash, the bacterial pellet was suspended in 150 μl of 4°C EPB, and stored on ice. 60 μl of the bacterial suspension was mixed with approximately 1μg of plasmid DNA and 375 μl of cold EPB. The suspension was placed in a chilled 0.2 cm gap sterile cuvette (Bio-Rad) and immediately electroporated, using a 2.5 kV pulse, 400 Ω, 25 μF. The bacterial solution was immediately pipetted into 1.0 ml of nonselective GCT media (for the *Pasteurellaceae*) or BHI broth (for all other bacteria), and incubated at 35°C for 90 minutes. A negative control, containing no plasmid DNA, was run in parallel with plasmid-containing bacterial solutions. Following incubation, the bacterial cultures were centrifuged in a microfuge for 3 minutes. The bacterial pellets were resuspended in approximately 90 μl of EPB (total volume of the solution was 100 μl). 20 μl of the electroporated cells were plated to each of 5 CA (for the *Pasteurellaceae*) or MacConkey agar (for *Proteus* sp.) or BAP (all other bacteria) containing the appropriate selective antibiotic. The negative controls were resuspended in EPB to a total volume of 100 μl, and 10 μl of the suspension was used to create 10^{-3}, 10^{-5} and 10^{-7} dilutions. 10 μl of the control suspension and the three dilutions were plated to the appropriate solid media with and without the selective antibiotic, to check for the development of spontaneous conversion to antibiotic resistance, and also to estimate the number of bacterial cells surviving the electroporation. All bacterial plates were incubated at 35°C in air, or, in the case of the *Pasteurellaceae*, in 5% CO_2. After a 24 hour incubation, the bacterial colonies from the negative control, nonselective plates were counted and the number of surviving cells determined. Selective plates were examined for the presence of transformed bacteria, colonies counted, and appropriate colonies were subcultured for plasmid screening.

I. DNA SEQUENCING

1. PREPARATION OF OLIGONUCLEOTIDE AND PLASMID DNA

Oligonucleotide sequences for pLS88, pHD148, pHD8.1, pPC100, pDM2, and pPW88 were taken from known RSF1010, Tn*916* and *cat* gene sequences. Primers were 18-22 bases in length, approximately 50% G-C in content, and were synthesized onto premade columns using the Applied Biosystems Inc. PCR-MATE 391 DNA Synthesizer. Following synthesis, the oligonucleotides were eluted from the columns using 3.0 ml of ammonium hydroxide over 2.5-3 hours. The eluted DNA was incubated overnight at 56°C to remove all protective groups from the nucleotides. Following incubation, the DNA solution was dried down in a speed vac (2-3 hours), and resuspended in 200 μl of deionized water. The concentration of the oligonucleotide in pmol/μl was calculated using the absorbance of a 1/500 dilution measured at 260 nm. It was assumed that 1 A_{260}= 30 μg/ml of single-stranded DNA, and that the average molecular weight of a base of DNA was 330 g/mol. A 3.2 pmol/μl dilution of oligonucleotide DNA in deionized water was needed for the sequencing reaction.

Plasmid DNA concentration was calculated using the absorbance of a dilution (1/200 to 1/500, as appropriate) measured at 260 nm. It was assumed that 1 A_{260}= 45 μg/ml of double stranded DNA. A 200 ng/μl dilution of double stranded plasmid DNA in TE was needed for the sequencing reaction.

2. SEQUENCING

DNA sequencing was accomplished by primer extension into unknown regions. Automated double stranded sequencing of both DNA strands was done *via* cycle sequencing, using the Applied Biosystems dye terminator system, and was done by Perry

D'Obrenan at the University of Alberta Biochemistry sequencing lab. Double stranded template DNA was mixed with the sequencing primer, 4 deoxynucleotide triphosphates (dATP, dTTP, dCTP, and dGTP), fluorescent dye-labeled dideoxynucleotide triphosphate sequence terminators (ddATP is green, ddTTP is red, ddCTP is blue, and ddGTP is yellow), sequenase, and buffer, according to the instructions of the manufacturer (Applied Biosystems, Inc.). All four sequencing reactions were done in one tube. The PCR cycle consisted of 1 minute (denaturation) at 94°C, 2 minutes (annealing) at 50°C, and 3 minutes (extension) at 72°C, for 30 cycles. After the last cycle, a final 10 minutes (extension) at 72°C was performed.

Following cycle sequencing, the reaction mixture was loaded onto a polyacrylamide gel, prepared according to the manufacturers instructions (Applied Biosystems, Inc.) and electrophoresed in the model 373A DNA automated sequencing system. The dye-labeled terminators were detected using scanning laser technology, and data collected on the system software (Applied Biosystems, Inc.).

Analysis of DNA sequence data was done using the IBM computer program PCGENE, release 6.7.

J. CREATION OF DNA-DNA HETERODUPLEXES AND ELECTRON MICROSCOPY

pHD148 DNA cut with *Sac*II was mixed with equal amounts of pHD8.1 DNA cut with *Sac*I, to a combined DNA concentration of 0.015 μg/ml. The DNA was denatured in 0.1 M NaOH, 20 mM EDTA (pH 12.5) in a 10 minute incubation at room temperature. Renaturation occurred in a 0.3 M Tris (pH 7.5) and 60% formamide over a three hour period. Cytochrome C was added to a final concentration of 50 μg/ml, and the DNA was spread onto a hypophase of 10% formamide in 10 mM Tris (pH 7.5), 1 mM EDTA. The DNA was picked up onto parlodion film attached to EM grids, stained with ethanolic uranyl acetate, rinsed with ethanol and isopentane, and allowed to dry. Following rotary shadowing with 20 Å platinum/carbon at an angle of 8° on a Balzers BA511M metal evaporation system, the DNA was viewed in a Philip's EM420 at 80 kV and photographs were taken with the combined STEM system (Davis, et al., 1971).

DNA lengths were measured using a computer-driven scanner apparatus. A total of 149 molecules were measured to ensure identity of the pictured DNA. The variance of molecules measured ranged from 117-285 map units, a +/- percentage error of 0.9-2.1%.

IV. RESULTS

A. PLASMID pLS88

1. HOST RANGE

The host range of pLS88 was suprisingly broad; the plasmid was successfully maintained in a variety of gram-negative hosts. pLS88 was not found to be stably maintained in the gram-positive hosts tested in this study. The host range and electroporation efficiency of pLS88 are given in Table 3.

Table 3. Transformation of bacteria with pLS88 by electroporation.

Bacterial Strains Transformed	Efficiency of Transformation[a]
Gram-negative	
Enterobacteriaceae	
Escherichia coli HB101	3.9×10^3
Salmonella typhimurium LT2	3.3×10^3
Proteus mirabilis (clinical isolate)	4.8×10^3

Pasteurellaceae	
Haemophilus influenzae Rd	6.2×10^2
Actinobacillus lignieressii ATCC 19393	7.2×10^2
Pasteurella multocida (clinical isolate)	2.7×10^3
Pseudomonaceae	
Pseudomonas aeruginosa DGI	8.3×10^2
Campylobacter	
Campylobacter jejuni (clinical isolate)	0
Helicobacter pylori (clinical isolate)	0
Gram-positive	
Streptococcaceae	
Streptococcus pneumoniae (clinical isolate)	0
Enterococcus faecalis ATCC 29212	0
Micrococcaceae	
Staphylococcus aureus ATCC 25923	0

[a] # transformants/μg DNA

2. SEQUENCES OF pLS88 AND pPC100

The size of pLS88 was 4,772 bp, and the deletion derivative, pPC100, was 1,838 bp. The complete nulceotide sequences of both pLS88 and pPC100 were determined and are given in Figure 7.

The sulfonamide and streptomycin A resistance genes of pLS88 (Figure 7, bases 108-899 and 944-1747, respectively) had nucleotide sequences with 99% homology to RSF1010 sequences. The protein translation sequence of the pLS88 sulfonamide

Figure 7. The complete nucleotide sequence of plasmids pLS88 and pPC100.

The sequence is numbered from the beginning of the *Pst*I site immediately upstream of the sulfonamide resistance gene. The sulfonamide, streptomycin A and kanamycin resistance genes are indicated by the encoded amino acid sequences below the nucleotide sequence line. Note that the streptomycin A resistance gene begins with a TTG start codon. Known promoters are bracketed, and the direction of transcription indicated by arrows. Unique areas of pLS88 and areas of pLS88 having homology with other plasmid sequences are indicated by dashed lines. The pLS88 *ori* region is highlighted by the icon "<". pPC100 sequence is bordered by arrows; the crossover points are highlighted by the icon "↓". An asterisk indicates a stop codon.

Figure 7. The complete nucleotide sequence of plasmids pLS88 and pPC100.

```
              .         .         .         .         .         .         .
   1 CTGCAGCTAATGGATCACCGCAAACAGGTTACTCGCCTGGGGATTCCCTTTCGACCCGAGCATCCGTATG
     ------ RSF1010 sul II upstream region -- ⌊P6  -35⌋ --> --------- ⌊P6

              .         .         .         .         .         .         .
  71 AGACTCATGCTCGATTATTATTATTATAGAAGCCCCCATGAATAAATCGCTCATCATTTTCGGCATCGTC
    -10⌋ --->  ------------ sul II gene-> M  N  K  S  L  I  I  F  G  I  V

              .         .         .         .         .         .         .
 141 AACATAACCTCGGACAGTTTCTCCGATGGAGGCCGGTATCTGGCGCCAGACGCAGCCATTGCGCAGGCGC
     N  I  T  S  D  S  F  S  D  G  G  R  Y  L  A  P  D  A  A  I  A  Q  A  R

              .         .         .         .         .         .         .
 211 GTAAGCTGATGGCCGAGGGGGCAGATGTGATCGACCTCGGTCCGGCATCCAGCAATCCCGACGCCGCGCC
       K  L  M  A  E  G  A  D  V  I  D  L  G  P  A  S  S  N  P  D  A  A  P

              .         .         .         .         .         .         .
 281 TGTTTCGTCCGACACAGAAATCGCGCGTATCGCGCCGGTGCTGGACGCGCTCAAGGCAGATGGCATTCCC
      V  S  S  D  T  E  I  A  R  I  A  P  V  L  D  A  L  K  A  D  G  I  P

              .         .         .         .         .         .         .
 351 GTCTCGCTCGACAGTTATCAACCCGCGACGCAAGCCTATGCCTTGTCGCGTGGTGTGGCCTATCTCAATG
     V  S  L  D  S  Y  Q  P  A  T  Q  A  Y  A  L  S  R  G  V  A  Y  L  N  D

              .         .         .         .         .         .         .
 421 ATATTCGCGGTTTTCCAGACGCTGCGTTCTATCCGCAATTGGCGAAATCATCTGCCAAACTCGTCGTTAT
       I  R  G  F  P  D  A  A  F  Y  P  Q  L  A  K  S  S  A  K  L  V  V  M

              .         .         .         .         .         .         .
 491 GCATTCGGTGCAAGACGGGCAGGCAGATCGGCGCGAGGCACCCGCTGGCGACATCATGGATCACATTGCG
      H  S  V  Q  D  G  Q  A  D  R  R  E  A  P  A  G  D  I  M  D  H  I  A

              .         .         .         .         .         .         .
 561 GCGTTCTTTGACGCGCGCATCGCGGCGCTGACGGGTGCCGGTATCAAACGCAACCGCCTTGTCCTTGATC
     A  F  F  D  A  R  I  A  A  L  T  G  A  G  I  K  R  N  R  L  V  L  D  P

              .         .         .         .         .         .         .
 631 CCGGCATGGGGTTTTTTCTGGGGGCTGCTCCCGAAACCTCGCTCTCGGTGCTGGCGCGGTTCGATGAATT
       G  M  G  F  F  L  G  A  A  P  E  T  S  L  S  V  L  A  R  F  D  E  L

              .         .         .         .         .         .         .
 701 GCGGCTGCGCTTCGATTTGCCGGTGCTTCTGTCTGTTTCGCGCAAATCCTTTCTGCGCGCGCTCACAGGC
      R  L  R  F  D  L  P  V  L  L  S  V  S  R  K  S  F  L  R  A  L  T  G

                                                               pPC100 -->
                                                                      ↓
              .         .         .         .         .         .         .
 771 CGTGGTCCGGGGATGTCGGGGCCGCGACACTCGCTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTT
     R  G  P  G  M  S  G  P  R  H  S  L  Q  S  L  P  P  P  Q  V  E  L  T  S

              .         .         .         .         .         .         .
 841 CATCCGCACACACGAGCCGCGCCCCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAACCGCA
       S  A  H  T  S  R  A  P  C  A  T  G  W  R  Y  W  R  R  *

              .         .         .         .         .         .         .
 911 AGAATTCGTTATCAGAAAACTGAAGGAACCTCCATTGAATCGAACTAATATTTTTTTTGGTGAATCGCAT
                         strA gene --> M  N  R  T  N  I  F  F  G  E  S  H

              .         .         .         .         .         .         .
 981 TCTGACTGGTTGCCTGTCAGAGGCGGAGAATCCGGTGATTTTGTTTTTCGACGTGGTGACGGGCATGCCT
     S  D  W  L  P  V  R  G  G  E  S  G  D  F  V  F  R  R  G  D  G  H  A  F

              .         .         .         .         .         .         .
1051 TCGCGAAAATCGCACCTGCTTCCCGCCGCGGTGAGCTCGCTGGAGAGCGTGACCGCCTCATTTGGCTCAA
       A  K  I  A  P  A  S  R  R  G  E  L  A  G  E  R  D  R  L  I  W  L  K
```

```
              .         .         .         .         .         .         .
1121 AGGTCGAGGTGTGGCTTGCCCCGAGGTGATCAACTGGCAGGAGGAACAGGAGGGTGCATGCTTGGTGATA
       G  R  G  V  A  C  P  E  V  I  N  W  Q  E  E  Q  E  G  A  C  L  V  I

              .         .         .         .         .         .         .
1191 ACGGCAATTCCGGGAGTACCGGCGGCTGATCTGTCTGGAGCGGATTTGCTCAAAGCGTGGCCGTCAATGG
      T  A  I  D  G  V  P  A  A  D  L  S  G  A  D  L  L  K  A  W  P  S  M  G

              .         .         .         .         .         .         .
1261 GGCAGCAACTTGGCGCTGTTCACAGCCTATCGGTTGATCAATGTCCGTTTGAGCGCAGGCTGTCGCGAAT
        Q  Q  L  G  A  V  H  S  L  S  V  D  Q  C  P  F  E  R  R  L  S  R  M

              .         .         .         .         .         .         .
1331 GTTCGGACGCGCCGTTGATGTGGTGTCCCGCAATGCCGTCAATCCCGACTTCTTACCGGACGAGGACAAG
       F  G  R  A  V  D  V  V  S  R  N  A  V  N  P  D  F  L  P  D  E  D  K

              .         .         .         .         .         .         .
1401 AGTACGCCGCAGCTCGATCTTTTGGCTCGTGTCGAACGAGAGCTACCGGTGCGGCTCGACCAAGAGCGCA
      S  T  P  Q  L  D  L  L  A  R  V  E  R  E  L  P  V  R  L  D  Q  E  R  T

              .         .         .         .         .         .         .
1470 CCGATATGGTTGTTTGCCATGGTGATCCCTGCATGCCGAACTTCATGGTGGACCCTAAAACTCTTCAATG
        D  M  V  V  C  H  G  D  P  C  M  P  N  F  M  V  D  P  K  T  L  Q  C

              .         .         .         .         .         .         .
1541 CACGGGTCTGATCGACCTTGGGCGGCTCGGAACAGCAGATCGCTATGCCGATTTGGCACTCATGATTGCT
       T  G  L  I  D  L  G  R  L  G  T  A  D  R  Y  A  D  L  A  L  M  I  A

              .         .         .         .         .         .         .
1611 AACGCCGAAGAGAACTGGGCAGCGCCAGATGAAGCAGAGCGCGCCTTCGCTGTCCTATTCAATGTATTGG
      N  A  E  E  N  W  A  A  P  D  E  A  E  R  A  F  A  V  L  F  N  V  L  G

              .         .         .         .         .         .         .
1681 GGATCGAAGCCCCCGACCGCGAACGCCTTGCCTTCTATCTGCGATTGGACCCTCTGACTTGGGGTTGATG
        I  E  A  P  D  R  E  R  L  A  F  Y  L  R  L  D  P  L  T  W  G  * |--

              .         .         .         .         .         .         .
1751 TTCATGCCGCCTGTTTTTCCTGCTCATTGGCACGTTTCGCAACCTGTTCTCATTGCGGACACCTTTTCCA
     ------------------------------------ strB gene ----------------------

              .         .         .         .         .         .         .
1821 GCCTCGTTTGGAAAGTTTCATTGACAGTAATACCAATGCTTTAGAAAGAAAAAGGGATCGAACTTTTGAC◄
     -----------------------|    |-----------------------------------------

              .         .         .         .         .         .         .
1891 ATTCGATCCCTTTTTCTGTAATCTGTTTCGTGCGTTCTTTGCTAAGATACAGACCCTAGACAAGTCATAT◄
     ----------------------------- ROB-1 β-lactamase, downstream region ---

              .         .         .         .         .         .         .
1961 CTTAGCAAAGGGTAGCTAGTAATGCAAGAGATTGCGAAGCGTCCCTACTACCAAAAAACCATTCAACGAC◄
     ------------------------------------------------------|

              .         .         .         .         .         .         .
2031 GTAAACAGACAAACGCAAACCTTAAATTAGACGGTCTTCAGCTCGGACTTCGGAAGAATAAACAGGCGTA◄

              .         .         .         .         .         .         .
2101 GAAGTGATAACGTTCTTAATACGAAAATTAAGCTCTGTCTCCGTTTCGTGCTACGGTTAGAAAGGCGAAA◄

              .         .         .         .         .         .         .
2171 GCCCCAAGAAATACAAGCACACCTGATAAGCGAGATTTAAGGATAACAGCGAAATTCAATAGGGTCTGAA◄

              .         .         .         .         .         .         .
2241 TTTCCAAACTAGGTTAAATGCCACGACGTTTTATTGTTGCCCCATTCAAGCAACATTTGAGAACCGAATA◄

              .         .         .         .         .         .         .
2311 GAAATCTTTTAGTAAAAAGCGTTCTTTTTTGGGTCAGCGGGTAATGTTGGACGGTTTAACGGTTTTTCCC◄
                                 |-------------------------------------
              .         .         .         .         .         .         .
2381 CTGCGGGTCGTATTGGAAAGCCATTGAAAAGCTGATGGATAACTCTGCGAGTTACCCACGAGCTTTCCAA<
     --------------------------- ROB-1 β-lactamase, complement of upstream
                   |----------------------------------

              .         .         .         .         .         .         .
2451 CAGCTTTCCAACACTAAAAACCTACCGCCCACAATAACCACTTCCCTAATAATAAAATTTTTTTCTTTTT<
     region -------|
     ----------------------------------------------------------------------

              .         .         .         .         .         .         .
2521 ATTTGGGTTCAAAGGCTCACGATGTTCGCCTAATAAAACGAAGTCGCCTATCGGCTCCGCTGATTTTTAT<
     --------------------- noncoding ORF ----------------------------------

              .         .         .         .         .         .         .
2591 ATATCACTCTCGGGGCTTTTGGTGTACTATTGTCTTTTGTAATAGCAAGGACACAAAAAGGGTACTCTTC<
     ----------------------------------------------------------------------

       pPC100 <--
              .  ↓      .         .         .         .         .         .
2661 GAGTTTCCTTTTTGACCTTGCAAAAGGGCTTTGCCCCCTTGACCCCCGACCGCTTTCAGCGGTCAAAATA<
     --------------|

              .         .         .         .         .         .         .
2731 GAAGAACGGACACCATTATGAAACGTGAGAAAGAGATAAAAATCAGGCTCACCGAAAACGAGTATCAAGC

              .         .         .         .         .         .         .
2801 CTTGTTAGAGAGAAAAACGAAAGCAAGGCTTGCGGAGTGGGTTCGGGAAGTTGCCCTGGAACAGCAACCT

              .         .         .         .         .         .         .
2871 AAGCGACAGCCGAAAGTAATCGACCCTGCGTTACTGTTCGAGCTGAACCGCATAGGCGTAAACCTGAACC

              .         .         .         .         .         .         .
2941 AAATCGCCCGACAATGCAACAGTCAAAAGCCGAGCATTGACCTTGTTAGCGTGTTGGCGACCTTGCATTG
                                                                      |----

              .         .         .         .         .         .         .
3011 CACAAGATAAAAATATATCATCATGAACAATAAAACTGTCTGCTTACATAAACAGTAATACAAGGGGTGT
     --------------- Tn903 sequence ------------------------- kan gene -->

              .         .         .         .         .         .         .
3081 TATGAGCCATATTCAACGGGAAACGTCTTGCTCGAGGCCGCGATTAAATTCCAACCTGGATGCTGATTTA
      M  S  H  I  Q  R  E  T  S  C  S  R  P  R  L  N  S  N  L  D  A  D  L

              .         .         .         .         .         .         .
3151 TATGGGTATAAATGGGCTCGCGATAATGTCGGGCAATCAGGTGCGACAATCTATCGATTGTATGGGAAGC
     Y  G  Y  K  W  A  R  D  N  V  G  Q  S  G  A  T  I  Y  R  L  Y  G  K  P

              .         .         .         .         .         .         .
3221 CCGATGCGCCAGAGTTGTTTCTGAAACATGGCAAAGGTAGCGTTGCCAATGATGTTACAGATGAGATGGT
       D  A  P  E  L  F  L  K  H  G  K  G  S  V  A  N  D  V  T  D  E  M  V

              .         .         .         .         .         .         .
3291 CAGACTAAACTGGCTGACGGCATTTATGCCTCTTCCGACCATCAAGCATTTTATCCGTACTCCTGATGAT
      R  L  N  W  L  T  A  F  M  P  L  P  T  I  K  H  F  I  R  T  P  D  D

              .         .         .         .         .         .         .
3361 GCATGGTTACTCACCACTGCGCTCCCCGGGAAAACAGCATTCCAGGTATTAGAAGAATATCCTGATTCAG
     A  W  L  L  T  T  A  L  P  G  K  T  A  F  Q  V  L  E  E  Y  P  D  S  G

              .         .         .         .         .         .         .
3431 GTGAAAATATTGTTGATGCGCTGGCAGCGTTCCTGCGCCGGTTGCATTCGATTCCTGTTAGTAATTGTCC
       E  N  I  V  D  A  L  A  A  F  L  R  R  L  H  S  I  P  V  S  N  C  P

              .         .         .         .         .         .         .
3501 TTTTAACAGCGATCGCGTATTTCGTCTCGCTCAGGCGCAATCACGAATGAATTACGGTTTGGTTGATGCG
      F  N  S  D  R  V  F  R  L  A  Q  A  Q  S  R  M  N  Y  G  L  V  D  A

              .         .         .         .         .         .         .
3571 AGTGATTTTGATGACGAGCGTAATGGCTGGCCTGTTGAACAAGTCTGGAAAGAAATGCATAAGCTTTTGC
     S  D  F  D  D  E  R  N  G  W  P  V  E  E  V  W  K  E  M  H  K  L  L  P
```

```
              .         .         .         .         .         .         .
3641 CATTCTCACCGGATTCAGACGTCACTCATGGTGATTTCTCACTTGATAACCTTATTTTTGACGAGGGGAA
       F  S  P  D  S  D  V  T  H  G  D  F  S  L  D  N  L  I  F  D  E  G  K

              .         .         .         .         .         .         .
3711 ATTAATAGGTTGTATTGATGTTGGACGAGTCGGAATCGCAGACCGATACCAGGATCTTGCCATCCTATGG
      L  I  G  C  I  D  V  G  R  V  G  I  A  D  R  Y  Q  D  L  A  I  L  W

              .         .         .         .         .         .         .
3781 AACTGCCTCGGTGAATTTTCTCCTTCATTACAGAAACGGCTTTTTCAAAAATATGGTATTGATAATCCTG
     N  C  L  G  E  F  S  P  S  L  Q  K  R  L  F  Q  K  Y  G  I  D  N  P  D

              .         .         .         .         .         .         .
3851 ATATGAATAAATTGCAGTTTCATTTGATGCTCGATGAGTTTTTCTGATAGTTAGTCTTTGTAGAGACACA
       M  N  K  L  Q  F  H  L  M  L  D  E  F  F  *  |----------------------

              .         .         .         .         .         .         .
3921 AACCTGAAATTCCGGACACCGCGCTTCAGGACATCTCCCTGGACGCCGATATCTGGCAGTATCCGGACGG
     --------------------------------------------------------- pLS88 unique

              .         .         .         .         .         .         .
3991 GACTATCGAACGCCGGGCCAACGGTACTCCCCTGCCCTTTATGACTGACGACCGGCTATTTCACCCACGC
     sequence -------------------------------------------------------------

              .         .         .         .         .         .         .
4061 ACTTCTCCATGAAATTTAGCATCGTCGTGTCACGCCCCCAAACCTGCAAACCCAGCAGGGGCGGGGGCTG
     ---------------------------------||-----------------------------------

              .         .         .         .         .         .         .
4131 GCGGGGTGTTGGAAAAATCCATCCATGATTATCCAAGAATAATCCACTAGGCGCGGTTATCAGCGCCCTT
     ---------------------- incomplete RSF1010 oriV sequence --------------
                                                     palindrome  |------>

              .         .         .         .         .         .         .
4201 GTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGCCAGAGGCCGGATAACTGATCTATCCGCTGCGCTAG
     ----------------------------------------------------------------------
        <------|                                                          |

              .         .         .         .         .         .         .
4271 GCTACACACCCGCCCCCACCGCTGCGCGGCAGGGGGAAAGGCGGGCAAAGCCCGCTAAACCCCACACCAA
     ----------------------------------------------------------------------
     --------------------------------ssiA ---------------------------->

              .         .         .         .         .         .         .
4341 ACCCGCAGTAATCCGCTGGAGCGATTTAGCCGCTTTAGCGGCCTTTTCCCTACCCGAAGGGGTGGGGGCG
     ----------------------------------------------------------------------
                       <--------------------------- ssiB ------------------
              .         .         .         .         .         .         .
4411 GTGTGCAGCCCCGAAGGGGCCAATTTAGCGGCCAATTTCAAGCGGCTTGTGATGCTGATAAGTGCCGTGG
     -----------||-------------------------- homology to pHD148 and pHD8.1
     -----------|

              .         .         .         .         .         .         .
4481 GTGGTCCCTTACGATATGGGAGTAATCAGGCTTGCCGAAAAGGGCTTTGAGTGGATCTGTCATGGCTGAT
     noncoding sequences --------------------------------------------------

              .         .         .         .         .         .         .
4551 CTCCAAACATATCAAGCGTGTCTGTATCCCACTCTATTGTAAACAAGACATTTTTATCTTTTATATTCAA
     --------------------------||------------------------------------------

              .         .         .         .         .         .         .
4621 TGGCTTATTTTCCTGCTAATTGGTAATATCATGAAAAATACCATGCTCAGAAAAGGCTTAACAATATTTT
     ------------------------- RSF1010 sul II upstream region -------------

              .         .         .         .         .         .         .
4690 GAAAAATTGCCTACTGAGCGCTGCCGCACAGCTCCATAGGCCGCTTTCCTGGCTTTGCTTCCAGATGTAT
     ----------------------------------------- ⌊P5  -35⌋ --> --------- ⌊P5
              .
4761 GCTATTCTGCTC
   -10⌋ --> ----
```

Figure 8. Protein translations of the antibiotic resistance genes of RSF1010, pLS88 and Tn*903*.

Panel A: RSF1010 published sulfonamide resistance gene translation (top line) and pLS88 predicted protein sequence (bottom line). Panel B: RSF1010 published streptomycin A resistance gene translation (top line) and pLS88 predicted protein sequence (Bottom line). Panel C: predicted kanamycin resistance gene translation from Tn*903* (top line) and pLS88 (bottom line). Sequence predictions were determined by nucleotide sequence analysis using the computer program PCGENE, release 6.4. The molecular mass of the proteins is given in Daltons. RSF1010 sequences were taken from Scholz, et al., 1989. Tn*903* sequences were taken from Oka, et al., 1981 and Grindley & Joyce, 1980.

Figure 8. Protein translations of the antibiotic resistance genes of RSF1010, pLS88 and Tn*903*.

```
               .         .         .         .         .         .         .
  A 1 MNKSLIIFGIVNITSDSFSDGGRYLAPDAAIAQARKLMAEGADVIDLVRHPAIPTPRLFRPTQKSRVCAG
      |||||||||||||||||||||||||||||||||||||||||||||||      |              | 
      MNKSLIIFGIVNITSDSFSDGGRYLAPDAAIAQARKLMAEGADVIDLGPASSNPDAAPVSSDTEIARIAP

               .         .         .         .         .         .         .
   71 AGRAQ-ADGIPVSLDSYQPATQAYALSRGVAYLNDIRGFPDAAFYPQLAKSSAKLVVMHSVQDGQADRRE
         |  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      VLDALKADGIPVSLDSYQPATQAYALSRGVAYLNDIRGFPDAAFYPQLAKSSAKLVVMHSVQDGQADRRE

               .         .         .         .         .         .         .
  141 APAGDIMDHIAAFFDARIAALTGAGIKRNRLVLDPGMGFFLGAAPETSLSVLARFDELRLRFDLPVLLSV
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      APAGDIMDHIAAFFDARIAALTGAGIKRNRLVLDPGMGFFLGAAPETSLSVLARFDELRLRFDLPVLLSV

               .         .         .         .         .
  211 SRKSFLRALTGRGPGVSGPRHSLQSLPPPQVELTSSAHTSRAPCATGWRYWRR       m.w. 28254
      ||||||||||||||| |||||||||||||||||||||||||||||||||||||
      SRKSFLRALTGRGPGMSGPRHSLQSLPPPQVELTSSAHTSRAPCATGWRYWRR       m.w. 28078


               .         .         .         .         .         .         .
  B 1 MNRTNIFFGESHSDWLPVRGGESGDFVFRRGDGHAFAKIAPASRRGELAGERDRLIWLKGRGVACPEVIN
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      MNRTNIFFGESHSDWLPVRGGESGDFVFRRGDGHAFAKIAPASRRGELAGERDRLIWLKGRGVACPEVIN

               .         .         .         .         .         .         .
   71 WQEEQEGACLVITAIPGVPAADLSGADLLKAWPSMGQQLGAVHSLSVDQCPFERRLSRMFGRAVDVVSRN
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      WQEEQEGACLVITAIPGVPAADLSGADLLKAWPSMGQQLGAVHSLSVDQCPFERRLSRMFGRAVDVVSRN

               .         .         .         .         .         .         .
  141 AVNPDLLPDEDKSTPLHDLLARVERELPVRLDQERTDMVVCHGDPCMPNFMVDPKTLQCSGLIDLGRLGT
      ||||| |||||||||  |||||||||||||||||||||||||||||||||||||||||| ||||||||||
      AVNPDFLPDEDKSTPQLDLLARVERELPVRLDQERTDMVVCHGDPCMPNFMVDPKTLQCTGLIDLGRLGT

               .         .         .         .         .
  211 ADRYADLALMIANAEENWAAPDEAERAFAVLFNVLGIEAPDRERLAFYLRLDPLTWG       m.w. 29529
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      ADRYADLALMIANAEENWAAPDEAERAFAVLFNVLGIEAPDRERLAFYLRLDPLTWG       m.w. 29568

               .         .         .         .         .         .         .
  C 1 MSHIQRETSCSRPRLNSNMDADLYGYKWARDNVGQSGATIYRLYGKPDAPELFLKHGKGSVANDVTDEMV
      |||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||
      MSHIQRETSCSRPRLNSNLDADLYGYKWARDNVGQSGATIYRLYGKPDAPELFLKHGKGSVANDVTDEMV

               .         .         .         .         .         .         .
   71 RLNWLTEFMPLPTIKHFIRTPDDAWLLTTAIPGKTAFQVLEEYPDSGENIVDALAVFLRRLHSIPVCNCP
      |||||| ||||||||||||||||||||||| |||||||||||||||||||||||| |||||||||| |||
      RLNWLTAFMPLPTIKHFIRTPDDAWLLTTALPGKTAFQVLEEYPDSGENIVDALAAFLRRLHSIPVSNCP

               .         .         .         .         .         .         .
  141 FNSDRVFRLAQAQSRMNNGLVDASDFDDERNGWPVEQVWKEMHKLLPFSPDSVVTHGDFSLDNLIFDEGK
      ||||||||||||||||| |||||||||||||||||||||||||||||||||||| |||||||||||||||||
      FNSDRVFRLAQAQSRMNYGLVDASDFDDERNGWPVEQVWKEMHKLLPFSPDSDVTHGDFSLDNLIFDEGK

               .         .         .         .         .         .
  211 LIGCIDVGRVGIADRYQDLAILWNCLGEFSPSLQKRLFQKYGIDNPDMNKLQFHLMLDEFF       m.w. 30979
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      LIGCIDVGRVGIADRYQDLAILWNCLGEFSPSLQKRLFQKYGIDNPDMNKLQFHLMLDEFF       m.w. 30924
```

resistance gene varied over a small region (Figure 8A) due to three one-base additions spaced over 84 bases. The streptomycin A protein sequences of pLS88 and RSF1010 had small variations (Figure 8B). An area homologous to the first 97 bases of the RSF1010 streptomycin B resistance gene was also present (Figure 7, bases 1748-1844). The sulfonamide-streptomycin A intergenic regions of RSF1010 and pLS88 were similar, with a 38 bp deletion of the RSF1010 sequence occurring at RSF1010 position 1-38 (Scholz, et al., 1989) in the pLS88 intergenic region.

The kanamycin resistance gene sequence (Figure 7, bases 3082-3897) showed 99% homology to that of Tn*903*, but lacked the terminal inverted repeats of the transposon (Grindley & Joyce, 1980; Oka, et al., 1981). A comparison between the pLS88 and Tn*903* kanamycin resistance protein sequences is given in (Figure 8C). The region between the kanamycin resistance gene and the sulfonamide resistance gene (Figure 7, bases 1-107 and 3898-4772) was not essential for plasmid replication or maintenance, as was shown by the pLS88 deletion derivative pPC100.

The sequence and structure of the *ori* was not found to be similar to those of known origins. Through the creation of pPC100, the *ori* was isolated to a region 1060 bp in length (Figure 7, bases 1845-2675). This region had few repeat sequences (16 bp inverted [Figure 7, bases 1866-1907, with a 9 bp loop] and 11 bp direct [Figure 7, bases 2441-2462]), one imperfect DnaA box (Figure 7, bases 2412-2422, inverted and complemented [Fuller, et al., 1984]) and two potential integration host factor (IHF) binding sites (Figure 7, bases 2497-2524 and 2583-2600). Included in the 1060 bases were sequences homologous to regions flanking the *H. influenzae* ROB-1 β-lactamase gene (Figure 7, bases 1849-2016 and complement of bases 2338-2465 [Juteau & Levesque, 1990; GenBank accession numbers M64762 and M64763]) and a small open reading frame (ORF; Figure 7, bases 2395-2675). It is not known whether the *H. influenzae* ROB-1 sequences are involved in replication.

3. PROTEIN PRODUCTION FROM pLS88 AND pPC100

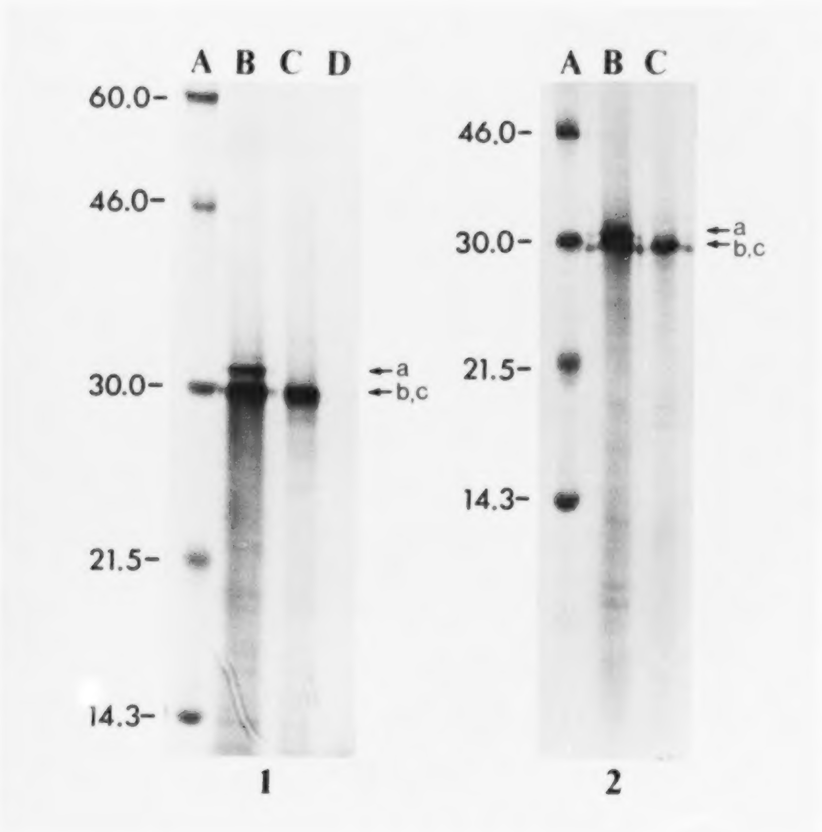
The possibility that pLS88 encoded proteins other than antibiotic resistance proteins was explored through *in vitro* transcription and translation of pLS88 and pPC100. *In vitro* studies failed to show the production of a protein from the ORF in the *ori* region of either plasmid (Figure 9).

Figure 9. *In vitro* translation of the plasmids pLS88 and pPC100.

Lane A contains ^{14}C-labelled protein molecular weight markers (Amersham). *In vitro* translation of pLS88 (lane B) and pPC100 (lane C) show the production of the ^{35}S-labelled kanamycin resistance gene product (a) and the streptomycin A and sulfonamide resistance gene products (b and c). No other protein products were evident. Lane D contains the product of an *in vitro* reaction in the absence of a template DNA, and serves as the negative control. The polyacrylamide concentrations of the gels were 12% (panel 1) and 20% (panel 2). The kanamycin resistance gene product from Tn*903* has a mass, in daltons, of 31,000 (Oka, et al., 1981; Grindley & Joyce, 1980); the streptomycin A and sulfonamide resistance gene products from RSF1010 have masses of 29,500 and 28,250, respectively (Scholz, et al., 1989). The mass of the protein product of the ORF was predicted to be 10,100.

The faint banding patterns seen in lanes A and B are consistent with background labeling of the *in vitro* reaction components.

Figure 9. *In vitro* translation of the plasmids pLS88 and pPC100.


A B C D
60.0-
46.0-
30.0-
21.5-
14.3-
←a
←b,c
1
A B C
46.0-
30.0-
21.5-
14.3-
←a
←b,c
2

B. PLASMIDS pHD8.1 AND pHD148

1. INCOMPATIBILITY

Using the RSF1010 insertion mutant AP101 (Tn*A* inserted in the sulfonamide resistance gene), which encodes an ampicillin resistance gene (Heffron, et al., 1975), compatibility between RSF1010, pHD148 and pHD8.1 was examined. Although the essential *rep* genes of RSF1010 appeared to be present in both pHD148 and pHD8.1, the region in RSF1010 containing the replication origin was deleted, raising questions about the replication mechanism in both plasmids. Electroporation of AP101 in *E. coli* containing pHD8.1 (and *vice versa*), with concurrent selection for both streptomycin (for pHD8.1) and ampicillin (for AP101) resistance yielded *E. coli* colonies with dual antibiotic resistance. These colonies were found to carry both plasmids. However, after serial passage of these colonies on nonselective media for 5 days, dual antibiotic resistance was not maintained, and only one plasmid could be found in any tested colony, suggesting incompatibility. Neither AP101 or pHD8.1 could be maintained forcibly with pHD148 in *E. coli*, due to the lack of a selective marker for pHD148. As a control, RSF1010 and AP101 were tested for incompatibility, and in the same manner, were found to be incompatible.

2. ELECTRON MICROSCOPY OF HETERODUPLEXES

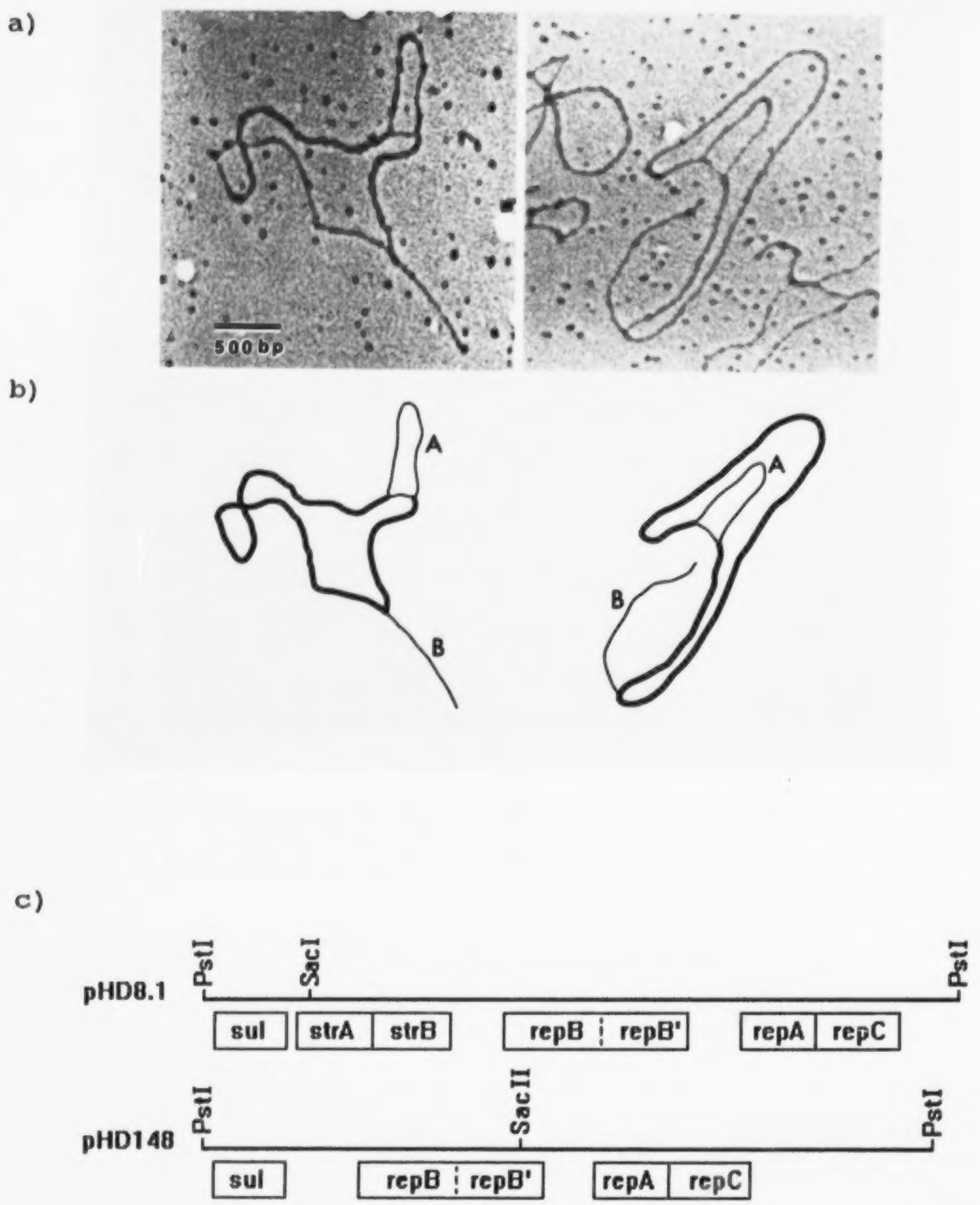
Because of the differences between RSF1010, pHD8.1 and pHD148 seen in restriction endonuclease digests and probing studies, the extent to which pHD148 and pHD8.1 were homologous was not fully known. In order to discover the degree of homology between the plasmids and minimize the amount of sequencing necessary to characterize differences between the two plasmids and RSF1010, DNA-DNA heteroduplexing of linear pHD148 and pHD8.1 was done. pHD8.1 digested with *Sac*I (cleaving at the 5' end of the streptomycin B resistance gene) was annealed to equal amounts of pHD148 digested with *Sac*II (cleaving near the 3' end of the *repB* gene) and examined using electron microscopy. The results are shown in Figure 10.

The long single-stranded region of the "A" loop seen in Figure 10 consists of an insertion loop in pHD148 downstream of the *repC* gene (see also map at bottom). The short single-stranded region seen in the "A" loop consists of an unknown sequence in pHD8.1, also downstream of the *repC* gene. The short double-stranded region between the loop and the single-stranded tail consists of the sulfonamide resistance gene in both plasmids. The single-stranded tail ("B" in Figure 10) consists of the streptomycin A and B resistance genes of pHD8.1. The long double-stranded region between the loop and

Figure 10. DNA-DNA heteroduplexes of the plasmids pHD148 and pHD8.1.

*Sac*II-linearized pHD148 (cleaving near the 3' end of the *repB* gene) was hybridized to *Sac*I-linearized pHD8.1 (cleaving near the 5' end of the streptomycin B resistance gene). (a) DNA-DNA heteroduplex between pHD148 and pHD8.1, magnification 86,500 x. (b) Line drawing illustrating pHD148 insertion loop (A) and the single-stranded tail at the pHD8.1 streptomycin resistance gene sequences (B). The tail is generated by cleavage of pHD8.1 at the *Sac*I site at the 5' end of the streptomycin B resistance gene. (c) Physical maps of pHD148 and pHD8.1, linearized at the *Pst*I sites upstream of the sulfonamide resistance genes, and showing the locations of genes relative to the *Sac*I and *Sac*II restriction endonuclease sites.

Figure 10. DNA-DNA heteroduplexes of the plasmids pHD148 and pHD8.1.


a)
500 bp
b)
A
B
A
B
c)
pHD8.1
PstI
SacI
PstI
sul
strA
strB
repB
repB'
repA
repC
pHD148
PstI
SacII
PstI
sul
repB
repB'
repA
repC

the single-stranded tail contains the *repB/A/C* region of both plasmids. The only significant regions of non-homology between pHD148 and pHD8.1 appear to be between the *repC* and sulfonamide resistance gene, and between the sulfonamide resistance gene and the *repB* gene.

3. SEQUENCES OF pHD8.1 AND pHD148

Sequencing of the variant regions of pHD8.1 and pHD148 revealed some very interesting features, including homology between RSF1010 (Scholz, et al., 1989), pHD8.1, pHD148, and pLS88. Figure 11 compares the sulfonamide-streptomycin A intergenic regions of RSF1010, pHD8.1, pHD148 and pLS88. Figure 12 demonstrates the deletion of the RSF1010 *oriV* region in both pHD148 and pHD8.1. Figure 13 demonstrates the sequence additions downstream of *repC* in pHD148 and pHD8.1 *vs* RSF1010, and Figure 14 compares RSF1010 *oriV*-like sequences in pHD148 and pHD8.1. Figure 15 shows a pictoral representation of the sequence data presented in Figures 11-14.

Figure 11. Nucleotide sequence comparisons of the sulfonamide-streptomycin A intergenic regions of the plasmids RSF1010, pHD8.1, pLS88 and pHD148.

The sequences begin at the *Pst*I site immediately downstream of the sulfonamide resistance gene and end at the ATT start codon of the streptomycin A resistance gene.

The comparison illustrates the 38 bp deletions common to the plasmids pHD8.1 and pLS88 relative to RSF1010. The 5' crossover point for the deletion of the streptomycin resistance genes of pHD148 is also shown.

RSF1010 sequences are from Scholz, et al., 1989.

Figure 11. Nucleotide sequence comparisons of the sulfonamide-streptomycin A intergenic regions of the plasmids RSF1010, pHD8.1, pHD148 and pLS88.

```
                      .         .         .         .         .         .
RSF1010      CTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACACGAGCCGCGCC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pHD8.1       CTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACACGAGCCGCGCC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pLS88        CTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACACGAGCCGCGCC
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pHD148       CTGCAGAGCTTGCCGCCGCCGCAGGTGGAGCTGACTTCATCCGCACACACGAGCCGCGCC

                       .         .         .         .         .         .         .
61    CCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAACCGCAAGAATTCGTTAACTGCACATTCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      CCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAACCGCAAGAATTCGTTA------------
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      CCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAACCGCAAGAATTCGTTA------------
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      CCTTGCGCGACGGGCTGGCGGTATTGGCGGCGCTGAAAGAAACCGCAAGAATTCGTTAACTGCACATTCG

                       .         .         .         .         .
131   GGATATTTCTCTATATTCGCGCTTCATCAGAAAACTGAAGGAACCTCCATT
      ||||||||||||||||||||| |||||||||||||||||||||||||||||
      --------------------------TCAGAAAACTGAAGGAACCTCCATT
                                |||||||||||||||||||||||||
      --------------------------TCAGAAAACTGAAGGAACCTCCATT
      ||||||||||||||||||||| ||||
      GGATATTTCTCTATATTCGCGGTTCA-------------------------
```

Figure 12. Nucleotide sequence comparisons of the *oriV* regions of plasmids RSF1010, pHD8.1 and pHD148.

The sequences begin at the end of the streptomycin B resistance gene at the *Eco*RV site and ends at the *repB* gene.

The RSF1010 *oriV* is bracketed by a solid line, from position 647-1044. The direct repeats of the origin are highlighted by solid arrows; dashed lines indicate the positions of the *ssiA* and *ssiB* replication initiation sites. The comparison illustrates the deletion of the regions of pHD148 and pHD8.1 corresponding to the RSF1010 plasmid *oriV*.

The *oriT* of RSF1010 is bracketed by a solid line from positions 1388-1478. Repeat sequences are highlighted by long dashed and short dashed lines. The comparison illustrates the homology of the *oriT* region in both pHD8.1 and pHD148, and the differences between these plasmid sequences and the RSF1010 sequence in this region.

RSF1010 sequences are from Scholz, et al., 1989.

Figure 12. Nucleotide sequence comparisons of the *oriV* regions of plasmids RSF1010, pHD148, and pHD8.1.

```
                   .         .         .         .         .         .
RSF1010   GATATCAAGCGACTTCTCCTATCCCCTGGGAACACATCAATCTCACCGGAGAATATCGCT
          ||||||||||||||||||||||||||||||||||||||||||| |||||||||||||| |
pHD8.1    GATATCAAGCGACTTCTCCTATCCCCTGGGAACACATCAATCTTACCGGAGAATATCGTT
pHD148    ------------------------------------------------------------

                   .         .         .         .         .         .         .
61        GGCCAAAGCCTTAGCGTAGGATTCCGCCCCTTCCCGCAAACGACCCCAAACAGGAAACGCAGCTGAAACG
          ||||||||||||||||||||||| |||||  ||||||||||||||||
          GGCCAAAGCCTTAGCGTAGGATTTCGCCCTCTCCCGCAAACGACCCC-----------------------
          ----------------------------------------------------------------------

                   .         .         .         .         .         .         .
131       GGAAGCTCAACACCCACTGACGCATGGGTTGTTCAGGCAGTACTTCATCAACCAGCAAGGCGGCACTTTC
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------

                   .         .         .         .         .         .         .
201       GGCCATCCGCCGCGCCCCACAGCTCGGGCAGAAACCGCGACGCTTACAGCTGAAAGCGACCAGGTGCTCG
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------

                   .         .         .         .         .         .         .
271       GCGTGGCAAGACTCGCAGCGAACCCGTAGAAAGCCATGCTCCAGCCGCCCGCATTGGAGAAATTCTTCAA
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------

                   .         .         .         .         .         .         .
341       ATTCCCGTTGCACATAGCCCGGCAATTCCTTTCCCTGCTCTGCCATAAGCGCAGCGAATGCCGGGTAATA
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------

                   .         .         .         .         .         .         .
411       CTCGTCAACGATCTGATAGAGAAGGGTTTGCTCGGGTCGGTGGCTCTGGTAACGACCAGTATCCCGATCC
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------

                   .         .         .         .         .         .         .
481       CGGCTGGCCGTCCTGGCCGCCACATGAGGCATGTTCCGCGTCCTTGCAATACTGTGTTTACATACAGTCT
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------

                   .         .         .         .         .         .         .
551       ATCGCTTAGCGGAAAGTTCTTTTACCCTCAGCCGAAATGCCTGCCGTTGCTAGACATTGCCAGCCAGTGC
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------

                   .         .        <------------------>  <------------------>
621       CCGTCACTCCCGTACTAACTGTCACGAACCCCTGCAATAACTGTCACGCCCCCCTGCAATAACTGTCACG
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------
                                   |───────────────────────────────────────────

            <------------------>       .         .         .         .         .
691       AACCCCTGCAATAACTGTCACGCCCCCAAACCTGCAAACCCAGCAGGGGCGGGGCTGGCGGGGTGTTGG
          ----------------------------------------------------------------------
          ----------------------------------------------------------------------
          ──────────────────────────────────────────────────────────────────────
```

```
               .         .         .         .         .         .         .
761   AAAAATCCATCCATGATTATCTAAGAATAATCCACTAGGCGCGGTTATCAGCGCCCTTGTGGGGCGCTGC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------
      ──────────────────────────────────────────────────────────────────────

               .         .         .         .         .        ────────────
830   TGCCCTTGCCCAATATGCCCGGCCAGAGGCCGGATAGCTGGTCTATTCGCTGCGCTAGGCTACACACCGC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------
      ─────────────────────────── RSF1010 oriV ─────────────────────────────

      ───────────────────────────────────────────────────>       .         .
901   CCCACCGCTGCGCGGCAGGGGAAAGGCGGGCAAAGCCCGCTAAACCCCACACCAAACCCCGCAGAAAT
      --------------------------------------------------------------------
      --------------------------------------------------------------------
      ────────────────────────────────────────────────────────────────────

            <────────────────────────────────────────────────────────────────
971   ACGCTGGAGCGCTTTTAGCCGCTTTAGCGGCCTTTCCCCCTACCCGAAGGGTGGGGGCGCGTGTGCAGC
      ---------------------------------------------------------------------
      ---------------------------------------------------------------------
      ─────────────────────────────────────────────────────────────────────

      ──       .         .         .         .         .         .         .
1041  CCCGCAGGGCCTGTCTCGGTCGATCATTCAGCCCGGCTCATCCTTCTGGCGTGGCGGAGACCGAACAAG
                                                   || | |||||  || ||||||| |
      ---------------------------------------------CTTGGGTGGC--AGTCCGAACACG
                                                    |||||||||||||||||||||||
      ----------------------------------------------TTGGGTGGC--AGTCCGAACACG
      ──|

               .         .         .         .         .         .         .
1111  GCGCGGTCGTGGTCGCGTTCAAGGTACGCATCCATTGCCGCCATGAGC---CGATCCTCCGGCCACTCGC
      || || ||  ||||||| || ||||| ||   |    ||  |  ||||   || |  || ||||||||||
      GCCCGATCACGGTCGCGCTCTAGGTA-GCTATCCAACCCATC--GAGCAGGCGCTGTTCTGGCCACTCGC
      ||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      GCCCGATCATGGTCGCGCTCTAGGTA-GCTATCCAACCCATC--GAGCAGGCGCTGTTCTGCCCACTCGC

               .         .         .         .         .         .         .
1181  TGCTGTTCACCTTGGCCAAAATCATGGCCCCCACCAGCACCTTGCGCCTTGTTTCGTTCTTGCGCTCTTG
       ||| |  |||||| | |  |||   |  || |||||||||||||||   || || | |||||||| |||
      -GCTTTCAACCTTGCCGAGTATCCACGTGCCTACCAGCACCTTGCGCGG-GTGTCCTCCTTGCGCTTTTG
      ||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
      -GCTTTCAACCTTGCCGAGTATCCACGCGCCTACCAGCACCTTGCGCGG-GTGTCCTCCTTGCGCTTTTG

               .         .         .         .         .         .         .
1251  CTGCTGTTCCCTTGCCCGCACCCGCTGAATTTCGGCATTGATTCGCGCTCGTTGTTCTTCGAGCTTGGCC
      |||||| |||| |||||  || ||||| || ||||| |||||||| || ||||| ||||| ||||| ||
      CTGCTGCTCCCGTGCCCTGACTCGCTGTATCTCGGCGTTGATTCGTGCCCGTTGCTCTTCTAGCTTTGCG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      CTGCTGCTCCCGTGCCCTGACTCGCTGTATCTCGGCGTTGATTCGTGCCCGTTGCTCTTCTAGCTTGGCG

               .         .         .         .         .         .         .
1321  AGCCGATCCGCCGCCTTGTTGCTCCCCTTAACCATCTTGACACCCCA-TTGTTAATGTGCTGTCTCGTAG
      || || ||||| || ||               ||| ||||||  ||  ||| | |||||||  |   ||
      AGTCGTTCCGCTGCTTTTGA------------CATGTTGACAATCCTCTTGGTTATGTGCTTGCCATTAT
      ||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
      AGTCGTTCCGCTGCTTTTGA------------CATATTGACAATCCTCTTGGTTATGTGCTTGCCATTAT
                                                                         |──
               .         .         ───────────→ ←──────────         --------
1391  GCTATCATGGAGGCACAGCGGCGGCAATCCC-GACCCTACTTTGTAGGGGAGGGCGCACTTACCGGTTTC
      |||||||| |  |||||   || |||| ||| |||| |  ||   | |||||||||||||||||||||||
      GCTATCATCGGAGCACATACGCCGCAAACCCCGACCGTCATTCTGACGGGAGGGCGCACTTACCGGTTTC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      GCTATCATCGGAGCACATACGCCGCAAACCCCGACCGTCATTCTGACGGGAGGGCGCACTTACCGGTTTC
      ──────────────────────────── RSF1010 oriT ────────────────────────────

      →    ←--------
1461  TCTTCGAGAAACTGGCCTAACGGCCACCC---TTCGGGCGGTGCGCTCTCTCCGAGGGCCATTGCATGGA
      |||||||||||||| |||  |||  ||||   || |||| |||||||||  |||||||||||||||||||
      TCTTCGAGAAACTGCCCT-GCGGG-ACCCCCTTTGGGGCTGTGCGCTCT--CCGAGGGCCATTGCATGGA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TCTTCGAGAAACTGCCCT-GCGGG-ACCCCCTTTGGGGCTGTGCGCTCT--CCGAGGGCCATTGCATGGA
      ─────────────────|

               .         .         .
1531  GCCGAAAAGCAAAAGCAACAGCGAGGCAGCATG
      ||   ||| |                    |||
      GC--TAAACCCC------------------ATG
      |||||||||||| ||||||||||||||||||||
      GC--TAAACCCCT-----------------ATG
```

Figure 13. Nucleotide sequence comparisons of the region 5' to the *repC* gene of plasmids RSF1010, pHD148, pHD8.1 and pLS88.

The sequences begin at the end of the *repC* gene at the *Bst*EII site and end at the *Pst*I site immediately upstream of the sulfonamide resistance gene.

The comparison illustrates insertions downstream of the *repC* gene in pHD148 and pHD8.1 relative to the RSF1010 sequence. The sequence in the insertion at postion 162-516 is homologous to nonessential pLS88 sequence downstream of the kanamycin resistance gene (see Figures 3 and 7). Solid arrows indicate a stem loop structure in the pHD148 insertion loop with a high GC content and near perfect homology; the N's are present due to an inability to sequence through the stem structure.

Sequence at position 1785-1922 are homologous between pHD148, pHD8.1, and pLS88 but are not present in RSF1010. In pLS88 this sequence is upstream of the sulfonamide resistance gene and is nonessential for plasmid replication or maintenance.

RSF1010 sequence is from Scholz, et al., 1989.

Figure 13. Nucleotide sequence comparisons of the region 5' to the *repC* gene of plasmids RSF1010, pHD148, pHD8.1 and pLS88.

```
                       .         .         .         .         .         .
RSF1010       GGTAACCGAGTTCGCGGCGGGCAAGTACGACATCACCCGGCCCAAGGCGGCAGGCTGACC
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
pHD148        GGTAACCGAGTTCGCGGCGGGCAAGTACGACATCACCCGGCCCAAGGCGGCAGGCTGACC
              ||||||||||| | | ||||||||||||||||| ||||||||||||||||||| ||||||
pHD8.1        GGTAACCGAGTACCCCGCGGGCAAGTACGACATTACCCGGCCCAAGGCGGCAGACTGACC
pLS88         ------------------------------------------------------------

             .         .         .         .         .         .         .
61  CCCCC-----------------------------------------------------------------
    |||||
    CCCCCTGTTTAACTGTCACGCCCCCCCCGATAACTGTCACGCCCCCCCCGAT-AACTGTCACGCCCCCCC
    |||| |   ||||||||||||||||  | ||||| ||||||  ||||| | | |||||||||||||||||
    CCCCGTAC-TAACTGTCACGCCCCCTGCAATAACAGTCACGAACCCCCTGTTTAACTGTCACGCCCCCCC
    ----------------------------------------------------------------------

             .         .         .         .         .         .         .
131 ----------------------------------------------------------------------
    CCGATAACTGTCACGAACCCCCCCGATAACTGTCACGCCCCCAAACCTGCAAACCCAGCAGGGGCGGGGG
        ||||||||||||||||||    ||||||||||||||||||||||||||||||||||||||||||||
    TGTTTAACTGTCACGAACCCCCTGTTTAACTGTCACGCCCCCAAACCTGCAAACCCAGCAGGGGCGGGGG
                                   |||||||||||||||||||||||||||||||||||||||
    -------------------------------GTCACGCCCCCAAACCTGCAAACCCAGCAGGGGCGGGGG

201 ----------------------------------------------------------------------
    CTGGCGGGATGTTGGAAAAATCCATCCATGATTATCCAAGAATAATCCACTAGGCGCGGTTATCAGCGCC
    |||||||| |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    CTGGCGGGGTGTTGGAAAAATCCATCCATGATTATCCAAGAATAATCCACTAGGCGCGGTTATCAGCGCC
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    CTGGCGGGGTGTTGGAAAAATCCATCCATGATTATCCAAGAATAATCCACTAGGCGCGGTTATCAGCGCC

             .         .         .         .         .         .         .
271 ----------------------------------------------------------------------
    CTTGTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGCCAGAGGGCGGATAACTGATCTATCCGCTGCGC
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    CTTGTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGCCAGAGGCCGGATAACTGATCTATCCGCTGCGC
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    CTTGTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGCCAGAGGCCGGATAACTGATCTATCCGCTGCGC

         <----------------------------------         .         .         .
341 ----------------------------------------------------------------------
    TAGGCTACACACCCGCCCCCACCGCTGGGCGGCAGGGGGAA-----------------------------
    |||||||||||||||||||||||||||||||||||||||||
    TAGGCTACACACCCGCCCCCACCGCTGCGCGGCAGGGGGAAAGGCGGGCAAAGCCCGCTAAACCCCACAC
    ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
    TAGGCTACACACCCGCCCCCACCGCTGCGCGGCAGGGGGAAAGGCGGGCAAAGCCCGCTAAACCCCACAC

             .         .         .         .         .         .         .
411 ----------------------------------------------------------------------
    ----------------------------------------------------------------------
    CAAACCCGCAGTAATCCGCTGGAGCGATTTTAGCCGCTTTAGCGGCCTTTTCCCTACCCGAAGGG-TGGG
    |||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||| ||||
    CAAACCCGCAGTAATCCGCTGGAGCGATTT-AGCCGCTTTAGCGGCCTTTTCCCTACCCGAAGGGGTGGG

             .         .         .         .         .       _____________
481 ----------------------------------------------------------------------
    -----------------------------------NNNNNNNNNNNNNNNNNNNNNNCCCCCTGCCGCAC
    GGCGGTGTGCAGCCCCGAAGGGGCCAATTTAGCGCC-----------------------------------
    |||||||||||||||||||||||||||||||||||
    GGCGGTGTGCAGCCCCGAAGGGGCCAATTTAGCGGC-----------------------------------
```

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      ──────────────────────→       .         .         .         .         .
551   ----------------------------------------------------------------------
      AGCGGTGGGGGCGGGTGTGTAGCCCCGCAGGGGCCATTCTGACCGCCTTGCGCGTCGACATGGCGGCTGC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
      ----------------------------------------------------------------------
621   CCGTGTCTTTCGTCGCGGTGACCACCGCCATCGTGTCGTGATCGACAAGGATACGCGCCTGTCGGGCTAT
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
691   ----------------------------------------------------------------------
      ATGCTTGAACCCGCGCTCACAGCCGGTTTCACCTCGATGGGCATGGACGTATTCCTTTTTGGCCCGCTGC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
761   ----------------------------------------------------------------------
      CGACAACGTATAGGAAGAATAAACGCCCTTTTCACCCAAGTCCAACAGCTTTGGACCGCAGTTGACTCTT
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
831   ----------------------------------------------------------------------
      TCGACACCCCTGCGATGCAACCCAATCCGGCTGACGAGCTGAAACCCCAGAAGGTCAAGGGGGTGTCACT
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
901   ----------------------------------------------------------------------
      GACCGAGAAGGTGTTCGGAACCGTGGAGACGGTCGAAGGGGTGGCCCAAAGGTTGAACGCCAAAATAATG
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
971   ----------------------------------------------------------------------
      GGGTCTGTGCAGCCGATGNCCGAGAAANCCGCTGTAAGCGCCCAGAACGAGCGAAGAGCCAAGGAGCTAA
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1041  ----------------------------------------------------------------------
      GGGAAACCCTAGCCCAGCAGCAAAAACGCTTACAGGCCCTTCACCACGGCTTTAAGGGGCTATCCAAGGA
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1111  ----------------------------------------------------------------------
      TCAGGTAGCGGGACTGATCCGGCAGGCGGTGAAATTGCGGCAGGAGAACGAGCAGGAGAAGCAGGAGCGC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1181  ----------------------------------------------------------------------
      GCCCAGCAGATCAAAGAGCGATTCAAGGCCAAGCGGGAAACGGGAGCGCAGCGACCGCACAGGGGCCGTT
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1251  ----------------------------------------------------------------------
      AACCCTTTTCAACCAACTTTAACCAACCTTGAACATACCATAAACCATTGTTTAACCAACATTAAACCAC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------
```

```
               .         .         .         .         .         .         .
1321  ----------------------------------------------------------------------
      TTTCAACCAACCCTAAGCAACCGGGGTGTAAGGGCTGGCAGTAATCGCGCGGATTTACAGGCACGTCGGA
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1391  ----------------------------------------------------------------------
      TATGTTTACCAAAACTCCGTTTTTGACACATATGCCGTTCCAAAGGGTGCCCCGGGTAAACCCTCCCAGC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1461  ----------------------------------------------------------------------
      GCCCCGCTCAATCACGGGGGCAGGGTGACGCGGTGGGGTAGGTCGGGACTGGACCGTCCTCGTCCAGGA
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1531  ----------------------------------------------------------------------
      ACGCCACTTCGGCGCGTTCCAGGGTGCAGCCATCGAGGTTCAATTCTTCCATGTCCTGAGGGTGAAAACC
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1601  ----------------------------------------------------------------------
      CCCGCCTCCTCGCGGCTCTTGGTGTACCCGTAGCCCTTGGGTTTCCAGTACTCCAGACCTGTCTTGATGT
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1671  ----------------------------------------------------------------------
      AATACATGGTCATGGCCATACCTCGTCCTTGAGTTTCGATATAACGGAATCCAGCGCCGTTCTGGTGCCG
      ----------------------------------------------------------------------
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1741  ----------------------------------------------------------------------
      TCCAGCGTGTCTATACCCCTGTCGTAAGCCTCCAAAACAGCGGCCA-TTTCAGCGG-CT-GTGATGCTGA
                                                  ||||||||||||||||||||||||||
      --------------------------------------------CA-TTTCAGCGG-CT-GTGATGCTGA
                                                  || |||||   | || ||||||||||
      --------------------------------------------CAATTTCAAGCCGCTTGTGATGCTGA

               .         .         .         .         .         .         .
1811  ----------------------------------------------------------------------
      TAGTGGCCGTGG-TG-TCCCTTACGATATGGGAGTAATCAGGCTTGCCGAAAAGGGCTTTGAGTGGATCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TAGTGGCCGTGG-TG-TCCCTTACGATATGGGAGTAATCAGGCTTGCCGAAAAGGGCTTTGAGTGGATCT
      ||   ||||||| || ||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TAAGTGCCGTGGGTGGTCCCTTACGATATGGGAGTAATCAGGCTTGCCGAAAAGGGCTTTGAGTGGATCT

               .         .         .         .         .         .         .
1881  -----------------------------------------CACTCTATTGTAAACAAGACATTTTTATC
                                               |||||||||||||||||||||||||||||
      GTCATGGCTGATCTCCAAACATATCAAGCGTGTCTGTATCCCACTCTATTGTAAACAAGACATTTTTATC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      GTCATGGCTGATCTCCAAACATATCAAGCGTGTCTGTATCCCACTCTATTGTAAACAAGACATTTTTATC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      GTCATGGCTGATCTCCAAACATATCAAGCGTGTCTGTATCCCACTCTATTGTAAACAAGACATTTTTATC

               .         .         .         .         .         .         .
1951  TTTTATATTCAATGGCTTATTTCCTGCTAATTGGTAATACCATGAAAAATACCATGCTCAGAAAAGGCT
      |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
      TTTTATATTCAATGGCTTATTTCCTGCTCATTGGTAATACCATGAAAAATACCATGCTCAGAAAAGGCT
      |||||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||||||
      TTTTATATTCAATGGCTTATTTCCTGCTAATTGGTAATATCATGAAAAATACCATGCTCAGAAAAGGCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TTTTATATTCAATGGCTTATTTCCTGCTAATTGGTAATATCATGAAAAATACCATGCTCAGAAAAGGCT
```

```
                .         .         .         .         .         .         .
2021   TAACAATATTTTGAAAAATTGCCTACTGAGCGCTGCCGCACAGCTCCATAGGCCGCTTTCCTGGCTTTGC
       ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       TAACAATATTTTGAAAAATTGCCTACTGAGCGCTGCCGCACAGCTCCATAGGCCGCTTTCCTGGCTTTGC
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
       TAACAATATTTTGAAAAATTGCCTACTGAGCGCTGCCGCACAGCTCCATAGGCCGCCTTCCTGGCTTTGC
       |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||
       TAACAATATTTTGAAAAATTGCCTACTGAGCGCTGCCGCACAGCTCCATAGGCCGCTTTCCTGGCTTTGC

                .         .         .
2091   TTCCACATGTATGCTCTTCTGCTCCTGCAG
       ||||| ||||||||| ||||||||||||||
       TTCCAGATGTATGCTATTCTGCTCCTGCAG
       ||||||||||||||||||||||||||||||
       TTCCAGATGTATGCTATTCTGCTCCTGCAG
       ||||||||||||||||||||||||||||||
       TTCCAGATGTATGCTATTCTGCTCCTGCAG
```

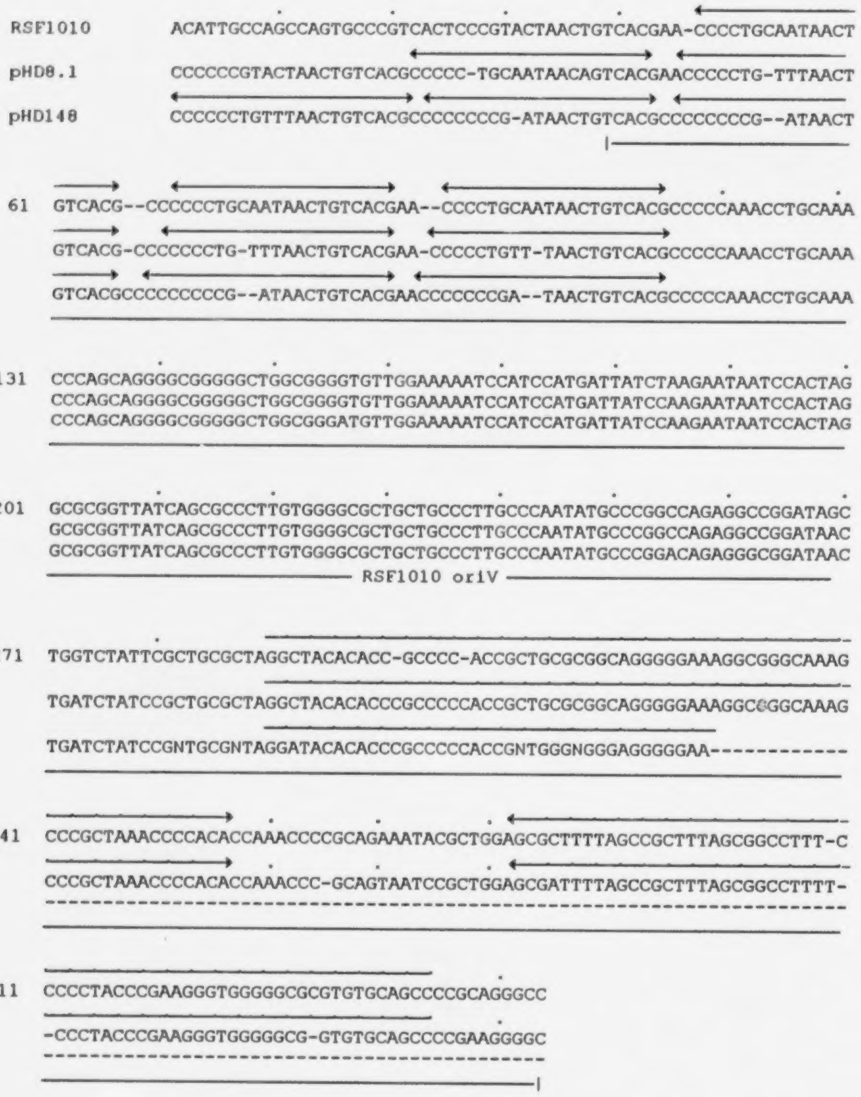
Figure 14. Nucleotide sequence comparisons of *oriV*-like regions in plasmids pHD148 and pHD8.1 and the RSF1010 *oriV*.

Sequences in pHD148 and pHD8.1 are immediately downstream of the *repC* gene. The RSF1010 *oriV* sequence is from Scholz, et al., 1989.

The RSF1010 *oriV* is bracketed by a solid line. Repeat sequences are highlighted by solid arrows. The *ssiA* and *ssiB* regions of RSF1010 and like regions in pHD8.1 and pHD148 are highlighted by notched arrows.

A comparison of the 20 bp repeats of the three plasmids is given at the bottom.

Figure 14. Nucleotide sequence comparisons of *oriV*-like regions in plasmids pHD148 and pHD8.1 and the RSF1010 *oriV*.



```
RSF1010     ACATTGCCAGCCAGTGCCCGTCACTCCCGTACTAACTGTCACGAA-CCCCTGCAATAACT
pHD8.1      CCCCCCGTACTAACTGTCACGCCCCC-TGCAATAACAGTCACGAACCCCCTG-TTTAACT
pHD148      CCCCCCTGTTTAACTGTCACGCCCCCCCCG-ATAACTGTCACGCCCCCCCCG--ATAACT

61   GTCACG--CCCCCCTGCAATAACTGTCACGAA--CCCCTGCAATAACTGTCACGCCCCCAAACCTGCAAA
     GTCACG-CCCCCCCTG-TTTAACTGTCACGAA-CCCCCTGTT-TAACTGTCACGCCCCCAAACCTGCAAA
     GTCACGCCCCCCCCCG--ATAACTGTCACGAACCCCCCCGA--TAACTGTCACGCCCCCAAACCTGCAAA

131  CCCAGCAGGGGCGGGGGCTGGCGGGGTGTTGGAAAAATCCATCCATGATTATCTAAGAATAATCCACTAG
     CCCAGCAGGGGCGGGGGCTGGCGGGGTGTTGGAAAAATCCATCCATGATTATCCAAGAATAATCCACTAG
     CCCAGCAGGGGCGGGGGCTGGCGGGATGTTGGAAAAATCCATCCATGATTATCCAAGAATAATCCACTAG

201  GCGCGGTTATCAGCGCCCTTGTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGCCAGAGGCCGGATAGC
     GCGCGGTTATCAGCGCCCTTGTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGCCAGAGGCCGGATAAC
     GCGCGGTTATCAGCGCCCTTGTGGGGCGCTGCTGCCCTTGCCCAATATGCCCGGACAGAGGGCGGATAAC
                                 RSF1010 oriV

271  TGGTCTATTCGCTGCGCTAGGCTACACACC-GCCCC-ACCGCTGCGCGGCAGGGGGAAAGGCGGGCAAAG
     TGATCTATCCGCTGCGCTAGGCTACACACCCGCCCCCACCGCTGCGCGGCAGGGGGAAAGGCGGGCAAAG
     TGATCTATCCGNTGCGNTAGGATACACACCCGCCCCCACCGNTGGGNGGGAGGGGGAA------------

341  CCCGCTAAACCCCACACCAAACCCCGCAGAAATACGCTGGAGCGCTTTTAGCCGCTTTAGCGGCCTTT-C
     CCCGCTAAACCCCACACCAAACCC-GCAGTAATCCGCTGGAGCGATTTTAGCCGCTTTAGCGGCCTTTT-
     ----------------------------------------------------------------------

411  CCCCTACCCGAAGGGTGGGGGCGCGTGTGCAGCCCCGCAGGGCC
     -CCCTACCCGAAGGGTGGGGGCG-GTGTGCAGCCCCGAAGGGGC
     --------------------------------------------
```

```
20 bp repeats:  RSF1010    CCCCTGCAATAACTGTCACG
                            ||||      ||||||||||| 
                pHD8.1     CCCCCTGTTTAACTGTCACG
                            |||||     |||||||||||
                pHD148     CCCCCCCGATAACTGTCACG
```

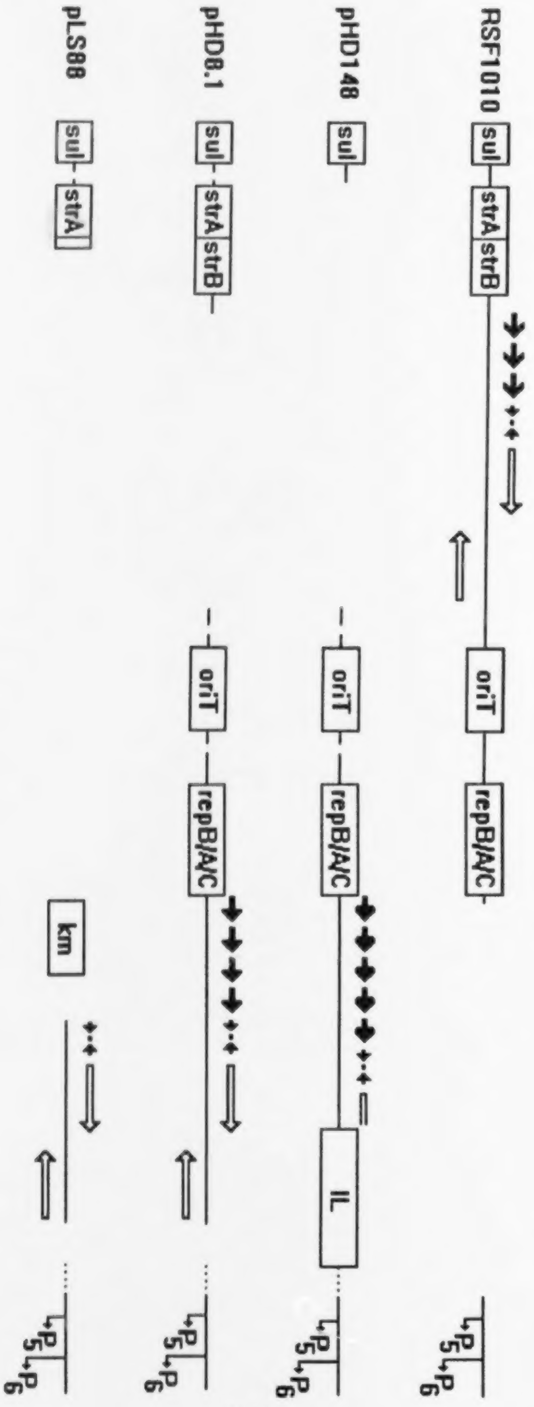
Figure 15. Sequence pictorals of plasmids RSF1010, pHD148, pHD8.1 and pLS88.

The map at the top represents the entire RSF1010 plasmid linearized upstream of the sulfonamide resistance gene. The large solid arrows represent the iterons of the *oriV*, the small solid arrows represent the palendromic sequence within the *oriV* region, and the open arrows represent the *ssi* sites. Promoter sites (P5 and P6) and direction of transcription are indicated. Other features are denoted by open boxes. Gaps have been inserted in the plasmid maps in order to line up features between plasmids.

Maps in the middle and at bottom represent plasmids pHD148, pHD8.1 and pLS88, oriented similarly to RSF1010. The differences in the large solid arrow shape in these plasmids represent differences in iteron sequences between plasmids. Solid lines indicate sequence homology with RSF1010. Small gaps in the solid lines seen in pHD148 and pHD8.1 represent small deletions from the RSF1010 sequence. Dotted lines indicate sequence homologies present but not shared with RSF1010.

Maps are not drawn to scale.

Figure 15. Sequence pictorals of plasmids RSF1010, pHD148, pHD8.1 and pLS88.


RSF1010
pHD148
pHD8.1
pLS88
sul
strA
strB
oriT
repB/A/C
km
IL
P5
P6

C. PLASMID pDM2

1. SEQUENCE OF pDM2

The sequence of the 5.6 kb *H. influenzae* plasmid RSF0885 was previously known, and is given in Figure 5. The sequence of the *Bam*HI fragment of the 55.1 kb, conjugative *H. influenzae* plasmid p2265 (inserted into RSF0885 to form pDM2) contains a chloramphenicol resistance gene with sequence homology to that of the large conjugative IncW *Shigella* plasmid pSa (Murray, et al., 1990). The chloramphenicol resistance gene sequence of pSa was used to create primers to sequence the remainder of the *Bam*HI insert.

The complete sequence of the *Bam*HI fragment of p2265, showing the chloramphenicol resistance gene and the locations and orientations of notable features, is given in Figure 16. The insert was found to be 3969 bp in length, and contained several interesting features, including three long, terminal repeat sequences, approximately 450 bp in length , two at one end in a direct orientation (Figure 16, bases 1-445 and 446-886), and the third on the other end in the opposite orientation (Figure 16, bases 3448-3969). Two groups of short, direct repeat sequences were also observed (Figure 16, bases 1200-1380).

Figure 16. Nucleotide sequence of the 4.1 kb *Bam*HI fragment of p2265 from plasmid pDM2.

The p2265 insert sequence of pDM2 is given, beginning and ending at the *Bam*HI sites, in the same orientation as is seen in Figure 4. The remainder of the pDM2 sequence, namely the RSF0885 sequence, is given in Figure 5.

Three repeated sequences were seen in the insert and are bracketed by dashed lines. Repeats #1 and #2 are direct; repeat #3 is inverted. The chloramphenicol resistance gene (*cat*) is bracketed by dashed lines; the start codon is at position 2211 and transcription proceeds leftward to position 1570. Short repeated sequences found in the insert are highlighted by dashed arrows.

Figure 16. Nucleotide sequence of the 4.1 kb *Bam*HI fragment of p2265 from plasmid pDM2.

```
                             .         .         .         .         .         .
    pDM2            GGATCCCGTTGACCGGTTTTATTTTTTACGGAACAGGGAGCGGAAATAATGGTCGCATCG
                    |-----------------------------------------------------------
                   .         .         .         .         .         .         .
      61  ATAATGATGCCCTGACGTAACATCAGTCCGGCCTGCTTCAGTTTCTGGTTAATCAGTGCCAGTATCTGTT
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     131  CTCCTGCATGATGTCTCTCCAGAAAATGGCGGAAATTCATGATGGTTGTGCGATCTGGCACCGTGTCACA
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     201  GATCCCCGCAAATTGACGGAACGGCAGGACATCATGCAGGGTGTCTTCAGCCGCTCCGTCACTGAGGTCA
          ------------------------------ repeat #1 -----------------------------
                   .         .         .         .         .         .         .
     271  AACCAGAGTTGGAGGCAGTGGATACGGAGCATAAGCACCAGGGGGGAAAGGCCGTCGGCCGCGAAGGGAT
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     341  GACGGATAGGACGTTAAAGCAGCTTTATCAGCAATATCCTGCCACGGCACCAGAACATTCATTCGGTCAA
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     411  GAAAACGGGGATTTACGGGAGGAATGCTTGGAAATGGATTCCCGTTGACCGGCTTTATTTTTTACGGAAC
          ----------------------------------|-----------------------------------
                   .         .         .         .         .         .         .
     481  AGGGAGCGGAAATAATGGTCGCATCGATAATGATGCCCTGACGTAACATCAGTCCGGCCTGCTTCAGTTT
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     551  CTGGTTAATCAGTGCCAGTATCTGTTCTCCTGCATGATGTTTCTCCAGAAAATGGCGGAAATTCATGATG
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     621  GTTGTGCGATCTGGCACCGTGTCACAGATCCCCGCAAATTGACGGAACGGCAGGACATCATGCAGGGCGT
          ------------------------------ repeat #2 -----------------------------
                   .         .         .         .         .         .         .
     691  CTTCAGCCGCTCCGTCACTGAGGTCAAACCAGAGTTGCAGGCAGTGGATACGGAGCATAAGCACCAGGGG
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     761  GAAAGGCCGTCGTCCGCGACGGGATGACGGATAGACGTGAAGCAGCTTATCAGCAATATCCTGCCACGGC
          ----------------------------------------------------------------------
                   .         .         .         .         .         .         .
     831  ACCAGAACATTCATACGGTCAAGAAAACGGGATTTACGGGAGGAATAGCTGGAAATGGCAGGCATTGTAA
          -------------------------------------------------------|
                   .         .         .         .         .         .         .
     901  ACAGCTCATGATAAAGAAAGCTTTATGTTCTCACATATGACGATTAAATCGCACCTTCCTTAGCAGATCG
                   .         .         .         .         .         .         .
     971  TCTTACTTTATTTATAACATCAAAAAATAAAACTAAAATATATACCTGTTCAGCGTAACTTATTATATAC
                   .         .         .         .         .         .         .
    1041  CTTCCTGATGAAGCCGGACGATATGTTTATACTGAACAGAAAGAATGCCAACCAGAATATTATCTTCTGT
                   .         .         .         .         .         .         .
    1111  TATACATGGCGGTTTATTTATTGTTTAATTAAACATATTCAGGCATATTTCTATACCATCATGATGTTTT
                   .         .         .         .         .         .         .
    1181  CACTGGTGTTACACCTGGTGAGTTAATACTAATGTTAATACTACAGGTGCGGTTACAGCATTCTGGTATT
                              |-------->  |-------->
                   .         .         .         .         .         .         .
    1251  GTTTAAAACGACTGTCGCAGCAGTCTTTCTGGTGTTGTAAATACTATTATTGTAAGTGCTATTACTATAA
                                              |--------------->>             |--
                   .         .         .         .         .         .         .
    1321  ATACTATTATTGTTATAACTGCATCTGGTGTTGTTACTAACTTATTACTGTTATTGTTACTGCTTTCTTT
          ------------->>                         |--------------->>
                   .         .         .         .         .         .         .
    1391  ATTACTATTGCTGTTCGTCTTTGCTTGTTTTTTCTTATTAAGGATATTACCAAACTACGGGAGCATTATC
                   .         .         .         .         .         .         .
    1461  GTACAGTGATCCTGAACCATTCTGAAACGAGATTACACATCACGACGAAAAGATAAAAAAAAGCCACCAT
                   .         .         .         .         .         .         .
    1531  TCTTCCGGATACAGTGGCTTAAATAAAGTATTAATTAACTTATTTCAGTATGTTATCACACATCATCTGA
                                                |-------------------------------
```

```
               .         .         .         .         .         .         .
1601  AGTGTATTAATAAACCTGGCTGCATGAAAGCCATCACAAACGGCATGATGTACCTGTACAGAAACAGGTA
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
1671  ATAATACGCGGTTATCTTCCTGCTGAAATTTCGCCATAGTAAACACCGGAGCAAAATAATCATCATTACC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
1741  GGTGATATTCAGGTTAAATCCGTCAAAACTCACCCAGGGTAATGATGATATATTCAGGTGGTTCTCTGGT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
1811  AACGCTCCCTGCGGGAACAATGCAGTATTATGCTGATATTCTGCCATCACCGCATTATAGCCCGCCATAA
      -------------------------------- cat ---------------------------------
               .         .         .         .         .         .         .
1881  ATTCACTGATATCCGGACAATAACGGCAGAAGAGCTCAGAAAATGTTTCAGTCTCTTTATGAAAAACAGT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
1951  AAATACAGGATCGGTCTGATCCCAGTAAATCAGTGCATTATCTTTCATTGCCATCCGGAACTCCGGAAAC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
2021  TGATTAACAACCCGGGAGATCAGATAAATCATCACCGGATAAAATTTATAATCCGTTTCCGCCAGTGCGG
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
2091  TACGAAAAGCTGTAATATCGAGTTTTGTGGTCAGGCTGAATCCGCATTTTATCTGCTGACGATAAAGAGC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
2161  AAAATGTTCTCTGCGGTTCCAGGTGTTCAGATCAATTCTGGTAAAATTCATGTTTATTCCTTCTGATTAA
      --------------------------------------------------|
               .         .         .         .         .         .         .
2231  TAGCGAAAAATATTAATAATCAGAAGGCAGTCTGGTTGTCTCAATGGGCAGCATCCCGTCCTCCGTAAGC
               .         .         .         .         .         .         .
2301  CGCTTGGTACAGAATAATGATATCAGATTCGGGCTCGCGAATTCAACTTCGAAGTGCAATTCCCGATTAA
               .         .         .         .         .         .         .
2371  AAATCAGGCTGCTTTCAGAAGTTCGTCAAAAACCACCTGTGGCTGTTTAAATCCCAAGCATTTGCGCGGC
               .         .         .         .         .         .         .
2441  CTGAAGTTCAGCCGCTTTTCGACGGCTGACAGTGCCTCTGCTGTCACCACACTCAAATCACTGGCTTTGG
               .         .         .         .         .         .         .
2511  GCACATATTGCCGCAGCAGACCATTGCTATTCTCATTCTTGTCCCCCGCTCCCATGCACTGTATTGGATG
               .         .         .         .         .         .         .
2581  CGCAAAGTACACCTGCGCTTCCAGCTCTGCTGCAACAACGTCCATGTTCGACAAACTCGCTACCACTGTC
               .         .         .         .         .         .         .
2651  AGCTGTGATGGAATGGTCATGTGGCTGGTAGGGTTTCAGCATGCTGATAATGGCGTCCGCCACCGCAGGT
               .         .         .         .         .         .         .
2721  GGTGGTGTTGGNTGGGTAACGTATAGAACCAGGTAGAGCCGGCTCTTACGCTCCAACAGACTCACTATTG
               .         .         .         .         .         .         .
2791  CACCGCTGCCTTGCTTACCCAACACGGTATCGACTTCCCAATCTCCCACACGGCTCCGCTCATTGACTAT
               .         .         .         .         .         .         .
2861  CGCAGGGCGTTCATCAATCGATACCGGATTGGGAATGACACTGCGTTTGCTGTTCTTACCCTTGCGATAG
               .         .         .         .         .         .         .
2931  CGTTTGTGACCCTGACGCAGATGCCGGTATAGCGTGCCACCGTTGGCCTTGTCGCGTGCCACATAGCGGT
               .         .         .         .         .         .         .
3001  ATATCCACTCGTGGCTAACCGGGCAGTTAATCATGTCTCCAACGCCACTGATTTGCTCGGGACTCCAGTC
               .         .         .         .         .         .         .
3071  CGCGACCAGCGCCATGCAAACAAAGTCGATGGTATCAGACGGGACAGTACGCTTCTGACTCGTCAGCCGT
               .         .         .         .         .         .         .
3141  CGTGCCGTGGCAAGTTGGTGAGCCTGCTGCGGCTGATAGGCCTGCCCAGCACAATTTACGACGAAGCTCA
               .         .         .         .         .         .         .
3211  CGACTGATAGTCGATGGATGAACCCCGAGCTTGTGTTGGCAATGCGGGCCTGTGACAGGCCATGGTCACG
               .         .         .         .         .         .         .
3281  GAGGCAGGCAATCTGGTATCGTAGCCCCTCGGTCAACTGCTGGTATTTCATGGTGTATCGCTTTGTTTCT
               .         .         .         .         .         .         .
3351  TTGGCGAGAAGAAGCGTACCCTAACTGGTAGTTGACCGCCTCTCTCCTATGCACCAGGGGCATTGCACTT
               .         .         .         .         .         .         .
3421  ATTATCTGAATTCACGCTTAAGATGTATTAAGGAAAGGTGCGATTTACTCGACATATGTGAGAACATAAA
                                 |------------------------------------------
               .         .         .         .         .         .         .
3491  GCTTTCTTTTATCATGAGCTGTTTTACAATGCCTGGCATTCCAGCTATTCCTCCCGTAAATCCCGTTTTC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
3561  TTGCCCGTATGAATGTTCTGGTCCCGTGGCAGGATATTTGCTGATAAGCTGCTTCACGTCCAATCCGTCA
      ----------------------------------------------------------------------
```

```
               .         .         .         .         .         .         .
3631  TCCCGTCGCGGCCCGACGGCCTTTCCCCCTGGTGCTTATGCTCCGTATCCACTGCCTGCAACTCTGGTTT
      ------------------------ repeat #3, reversed -------------------------
               .         .         .         .         .         .         .
3701  GACCTCAGTGACGGAGCGGCTGAAGACGCCCTGCATGATGTCCTGCCGTTCCGTCAATTTGCGGGGATCT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
3771  GTGACTCGGTGCCAGATCGCACAACCATCATGAATTTCCGCCATTTTCTGGAGAAACATCATGCAGGAGA
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
3841  ACAGATACTGGCACTGATTAACCAGAAACTGAAGCAGGCCGGACTGATGTTACGTCAGGGCATCATTATC
      ----------------------------------------------------------------------
               .         .         .         .         .
3911  GATGCGACCATTATTTCCGCTCCCTGTTCCGTAAAAAATAAAGCCGGTCAACGGGATCC
      ----------------------------------------------------------|
```

D. PLASMIDS pPW87 AND pPW88

1. SEQUENCES OF pPW87 AND pPW88

Both pPW87 and pPW88 were created from the 4.7 kb plasmid pLS88, through the replacement of the small (0.8 kb) *Pst*I fragment containing the sulfonamide resistance gene with the *Pst*I fragments containing the chloramphenicol resistance gene from the large, conjugative *Shigella* plasmids pSa and pSa*, respectively. The known sequence of the chloramphenicol resistance gene (Murray, et al., 1990) was used to create primers to sequence the remainder of the insert. Primers were also created from the pLS88 sequence bordering the insert.

The complete sequence of the *Pst*I insert from pPW87 is given in Figure 17, showing the positions and orientations of genes and repeat sequences. The insert was 3,379 bp in length. The 5' end of the insert contained the 5' end of the sulfonamide I resistance gene from In6 (Figure 17, bases 1-362), and the 3' end had two long, direct repeat sequences approximately 400 bp in length (Figure 17, bases 2486-2881 and 2882-3277), each containing a *Xho*I site (Figure 6). In pPW88, a third repeat is present in tandem with the other two. The first 27 bp of the repeats are duplicated immediately upstream of the first repeat in both pPW87 and pPW88.

Figure 17. Nucleotide sequence of the *Pst*I fragment of pSa from plasmids pPW87 and pPW88.

The pSa insert sequence is given in full, beginning and ending with the *Pst*I sites, in the orientation shown in Figure 6. The remainder of the pPW87 sequence is pLS88 sequence, seen in Figure 7.

The 5' end of the sulfonamide I sequence and the chloramphenicol resistance gene sequence of Int6 in pSa are bracketed by dashed lines. Repeat sequences of the integron are bracketed by dashed lines. Also given immediately below the pPW87 sequence for comparison is the sequence of the direct repeats of pSa (from Stokes, et al., 1993). Short repeat sequences are highlighted by dashed arrows.

The pSa* insert sequence of pPW88 contains a third repeat sequence (see Figure 6) identical to repeats #1 and #2, and is not shown.

Figure 17. Nucleotide sequence of the *Pst*I fragment of pSa from plasmids pPW87 and pPW88.

```
                         .         .         .         .         .         .
pPW87           CTGCAGGCCGCCTCAGCAATATCGGGATAGAGCGCAGGGTCAGGAAATCCTTGGATATCG
                <-----------------------------------------------------------

               .         .         .         .         .         .         .
  61  TTCAGGTAGGCCACGCCGCGCTTGAGCGCATAGCGCTGGGTTTCCCGTTGGGAGCTGTCGGTTGGAACAC
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
 131  GGGGCATCTTATCGNGCAGGGGTCTTAGGAGCGGGGAATACGTCTTATCTCATCGGCCGGCGATACAGG
      ---------------------- <-- sul I gene, 5' end <-- --------------------

               .         .         .         .         .         .         .
 201  CCTCGCGTCCGGATGGCTGGCGGCCGGTCCGACATCCACGACGTCTGATCCGACTCGCAGCATTTCGATC
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
 271  GCCGCGGTGACAGCGCCGGCGGGGTCTAGCCGCCGGCTCTCATCGAAAAGGGAGTCCTCGGTGAGATTCA
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
 341  GAATGCCGAACACCGTCACCATGGCGTCGGCCTCCGCAGCGACTTCCACGATGGGGATCGGGCGAGCAAA
      ---------------------|

               .         .         .         .         .         .         .
 411  AAGGCAGCAATTATGAGCCCCATACCTACAAAGCCCCACGCATCAAGCTTTTGCCCATGAAGCAACCAGG
               .         .         .         .         .         .         .
 481  CAATGGCTTTATTTTTGCCGACGCCGAGTCCCGACAAGACTTCATAAGCAACCCCGGCGGGGGGAGGATT
               .         .         .         .         .         .         .
 551  TCAGAACCAAGAGAACATTTTTTTTGTACTGGGAGGGCGCATTACCACCTTCGGCTGGGGGATGAGTGGC
               .         .         .         .         .         .         .
 621  ACNCCGTTTCTGCCGGGGNATCCCTGCGCTTCCATGCGGATATCCCGCACGCTTACGCCAATCCCGGTAA
               .         .         .         .         .         .         .
 691  GGCCATTGTGACACTGCATAATCTGATCCATTATCCGCGCCCGGCGGTCAAATAAAAAAGCAGGGGATAA
               .         .         .         .         .         .         .
 761  TAAATATACCCCGCTTTGACTTAACGGATCGTCTTACTTTATTTGTAAAATAAAACCCCNATAAATATGT
               .         .         .         .         .         .         .
 831  GTTCAGCTTAACTTATTATATATCATCCTTATACCAACCGGGATGATATGTTTATACTGAACAGAAAAGC
               .         .         .         .         .         .         .
 901  ATTCCATTCAGAATACTATGTTCTGTTATATATGGCGGTTTATTTATTGTTAATAACACACACTCAGGCA
               .         .         .         .         .         .         .
 971  TATCACTATGCTATCGTGATGTTTTCACTGTTGTTGTTACCTACTGCCTTTACGGCATTTTGTTGTTGTC
               .         .         .         .         .         .         .
1041  CCACAATGACTGTCGCAGCAGTCTTTCTGGTGTCTTAAATACTATTATTATAACTGCATCTGGTGTTGTT
               .         .         .         .         .         .         .
1111  AATATTATTGTTACTGCTTACTTTATTATTATTGCTGTCAGTCTTTGCTGTTTCTTTTTTATTAAGGGTA
               .         .         .         .         .         .         .
1181  TTACCACACTCCGGGGGCATTATCGTACAGTGATCCTGAACCAGTCTGAAACGAAATTACAGATTACGGG
               .         .         .         .         .         .         .
1251  TAAAATATAAAAAAAAGCCACCATTCCTGCCGGATACGGTGGCTTAAATACAGAATTAATTAATTTATTT
                                                                      |-----

               .         .         .         .         .         .         .
1321  CAGTATGTTATCACACATCAGCTGAAGTGTATTAATAAACCGTGCTGCATGAAAGCCATCACATACTGCA
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1391  TGATGAACCTGTACAGAAACAGGTAATAATACGCGGTCACCTTCCTGCTGAAACTTTGCCATAGTAAACA
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1461  CCGGAGCAAAATAATCATCATTACCGGTGATATTCAGGTTAAATCCGTCAAAACTCACCCACGGTAATGA
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1531  TGATATATTCAGGTGATTCTCCGGTAAATTTCCCTGCGGAAACAATCTGGTATCATGCTGATATTCTGCC
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1600  GTTACCGCATAATAACCTGCCATAAACTCACTGAGATCCGGAAAATAACGGCAGGACAGTGCAGAGAATG
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
1671  TTTCGGTTTCTTTATGAAAGACAGTAAAGACCGGGTCTGACTGGTCCCAGTAAATAAGTTCATTGTCTTT
      ----------------------------- <-- cat <-- ----------------------------
```

```
               .         .         .         .         .         .         .
1741  CAGTGCCATCCGGAACTCCTTAAACTGATTAACAGCCCGGGAGATCAGGTAAATCATCAGCGGATAAAAG
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
1811  TTATAACCTGTCTCCGCCAGTGCGGTACGCAAAGCGGTAATATCGAGTTTGGTGGTCAGGCTGAATCCGC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
1881  ATTTAATCTGCTGACGATAAAGGGCAAAGTGTTCCCTGCGATTCCAGGTATTCAGGTCAATCCGGGTAAA
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
1951  ATTCATGGTTATTCCTTCTGATTAATAGTGAAAAATATTAATAATCAGAAGGCAGTCTGGTTGTCTCAAT
      -----|
               .         .         .         .         .         .         .
2021  GGGTAACATTCCGTCCTCCGTAAGCTGTTTGGTATTCATTAATAATACCCTATACGGCTTAATCTGTAT
               .         .         .         .         .         .         .
2091  TAAGCCCGGCTTTATTTATTCCGGCCAATCATCCGCAAACACATAGCGGATCAGTTCTGCGGATTCACGG
               .         .         .         .         .         .         .
2161  GGCGGTGCTCTCAGCACATCCGCCATTAAATCAATCTCCATCTGACAGGTTTGCAGCTTGTCTTCCGCCG
               .         .         .         .         .         .         .
2231  GTACATACGGATCATCCGTCAGGAAACTATCGCCGTATTTATCAATCGACCCCTGTATTTGTGCCGAAAA
               .         .         .         .         .         .         .
2301  TAAAGTCACGGTACTCTCCGGAGGGCTTTTTCATATTCCGGCGGGGCTGACACTTCCGGATGCAGCACAC
               .         .         .         .         .         .         .
2371  GAAAACAGA-GTCACCGGAACACGCCATTCTGAGAAAACTGTCACTAATCTGTTTTATTCCGCAAACAGA
       ||| |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
       AAA-CAGAAGTCACCGGAACACGCCATTCTGAGAAAACTGTCACTAATCTGTTTTATTCCGCAAACAGA
               .         .         .         .         .         .         .
2441  AAACCACCGGATAACCGGGGTATAGGAAGTATAAACCACCTTTGGGGGTATAGGAAGTATAAACCACCTT
      |||||||||||||||||||||||||||||||||||||||||||  |||||||||||||||||||||||||
      AAACCACCGGATAACCGGGGTATAGGAAGTATAAACCACCTTTTTGGGTATAGGAAGTATAAACCACCTT
                                                   |------------------------
                       --------------------------->-------------------------
               .         .         .         .         .         .         .
2511  TGGGCTCCTCATCCGAAGTATCTTACCTGAAATTCCCTCACTCGTTTACCGCTCAAGCCCCAATTTTAAC
      |  |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TTTGCTCCTCATCCGAAGTATCTTACCTGAAATTCCCTCACTCGTTTACCGCTCAAGCCCCAATTTTAAC
      ----------------------------------------------------------------------
      -->
               .         .         .         .         .         .         .
2581  TGCCGGTCCAGCCTAAACCGCTCTAATAAGGTTCGATTTGGCGGTAAAATCTCTAGCCTGATAGCTCGAG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TGCCGGTCCAGCCTAAACCGCTCTAATAAGGTTCGATTTGGCGGTAAAATCTCTAGCCTGATAGCTCGAG
      -------------------------- repeat #1 ---------------------------------
               .         .         .         .         .         .         .
2651  AGATACAAACTGCCCCACCGCCCCGTTTAAAAGTTGGCAGTGTTGAGCAGTGTTGGATTTGGGGTCGTCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      AGATACAAACTGCCCCACCGCCCCGTTTAAAAGTTGGCAGTGTTGAGCAGTGTTGGATTTGGGGTCGTCA
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
2721  GTCAAAGAGACGACTCTGTGATGGATCGAACAGGCTGGGAGTCAGTGGCGGCGCTCGTTCTGGTGGCAGC
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||   ||||||
      GTCAAAGAGACGACTCTGTGATGGATCGAACAGGCTGGGAGTCAGTGGCGGCGCTCGTTCT---GGCAGC
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
2791  TCACGCTGCTTGGCGGCATTCGCCTTGGCTGTTTTCTGTTTCAGATGCTTGAGAATCTGCTCAATGACCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TCACGCTGCTTGGCGGCATTCGCCTTGGCTGTTTTCTGTTTCAGATGCTTGAGAATCTGCTCAATGACCT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
2861  TCGGATCTTCGATGCTGGCAAGGGTATAGGAAGTATAAACCACCTTTTTGCTCCTCATCCGAAGTATCTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      TCGGATCTTCGATGCTGGCAAGGGTATAGGAAGTATAAACCACCTTTTTGCTCCTCATCCGAAGTATCTT
      ---------------------|------------------------------------------------
                           --------------------------->
               .         .         .         .         .         .         .
2931  ACCTGAAATTCCCTCACTCGTTTACCGCTCAAGCCCCAATTTTAACTGCCGGTCCAGCCTAAACCGCTCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      ACCTGAAATTCCCTCACTCGTTTACCGCTCAAGCCCCAATTTTAACTGCCGGTCCAGCCTAAACCGCTCT
      ----------------------------------------------------------------------
               .         .         .         .         .         .         .
3001  AATAAGGTTCGATTTGGCGGTAAAATCTCTAGCCTGATAGCTCGAGAGATACAAACTGCCCCACCGCCCC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
      AATAAGGTTCGATTTGGCGGTAAAATCTCTAGCCTGATAGCTCGAGAGATACAAACTGCCCCACCGCCCC
      ----------------------------------------------------------------------
```

```
               .         .         .         .         .         .         .
3071  GTTTAAAAGTTGGCAGTGTTGAGCAGTGTTGGAATTGGGGTCGTCAGTCAAAGAGACGACTCTGTGATGG
      ||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
      GTTTAAAAGTTGGCAGTGTTGAGCAGTGTTGGATTTGGGGTCGTCAGTCAAAGAGACGACTCTGTGATGG
      ----------------------------- repeat #2 -------------------------------

               .         .         .         .         .         .         .
3141  ATCGAACAGNCTGGGAGTCAGTGTCGCCGCTCGTTCTGGTGACAGCTCACGCTGCTTGGCGGCATTCGCC
      ||||||||||||||||||||||| || ||||||||||||   ||||||||||||||||||||||||||||
      ATCGAACAGGCTGGGAGTCAGTGGCGGCGCTCGTTCTGG---CAGCTCACGCTGCTTGGCGGCATTCGCC
      ----------------------------------------------------------------------

               .         .         .         .         .         .         .
3211  TTGTCTGTTTTCTGTTTCAGATGCTTGAGAATCTGCTCAATGACCTTCGGATCTTCGATGCTGTCAATCA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||
      TTGGCTGTTTTCTGTTTCAGATGCTTGAGAATCTGCTCAATGACCTTCGGATCTTCGATGCTGGCAATCA
      ------------------------------------------------------------------|

               .         .         .         .         .         .         .
3281  CTTTGACGTGACCGCCGCAGTGTTCGCAGACTTCAATATCAATATTGAAGACTCGCTTGAGGCGTTGCAT
      |||||||||||||||||||||||||||||||||||||||      |||||||||||||||||||||||||
      CTTTGACGTGACCGCCGCAGTGTTCGCAGACTTCAATAT------TGAAGACTCGCTTGAGGCGTTGCAT

               .         .
3351  CCAGATCATGGCGCGGTGGCGCTCTGCAG
      |||| ||||| ||||||||||||||||||
      CCAGGTCATG-CGCGGTGGCGCTCTGCAG
```

IV. CONCLUSIONS AND DISCUSSION

A. PLASMID pLS88

1. SEQUENCE HOMOLOGIES WITH KNOWN PLASMIDS

The evolution of the sequence of pLS88 is quite complex, composed of sequences found in RSF1010, pHD148, pHD8.1, and the *H. influenzae* plasmid R_{Rob}. Other than the *ori* region, only a small region of pLS88, less than 200 bases in total, is unique (Figure 7, bases 3898-4094).

The sulfonamide II and streptomycin A resistance genes of pLS88 have nucleotide sequences with almost 100% identity to RSF1010 sequences (data not shown). The protein translation sequence of the pLS88 sulfonamide resistance gene varies over a small region (28 amino acids) from that of RSF1010 (Figure 8A) due to three one-base additions spaced over 84 bases (data not shown); sequences from the sulfonamide resistance plasmids pHD148 and pHD8.1 also contain the three one-base additions (data not shown). The streptomycin A protein sequences of pLS88 and RSF1010 have small variations (Figure 8B). An area homologous to the first 97 bases of the streptomycin B resistance gene are also present (Figure 7, bases 1748-1844).

It is possible that the sulfonamide-streptomycin A region of pLS88 is more related to that of pHD8.1 than RSF1010: the 38 base pair deletion from the RSF1010 sequence seen in the intergenic region of pLS88 is duplicated in pHD8.1 (Figure 11). In both of these plasmids, the RSF1010 promoters P5 and P6 are present (Figure 7) and initiate transcription of the sulfonamide and streptomycin resistance genes. The deletion in the intergenic region is such that the reading frame is not maintained.

A problem arises upon consideration of the pLS88 deletion derivative, pPC100. The region containing the P5 and P6 promoters is deleted, yet the streptomycin resistant phenotype remains (Figure 7). It is possible that the promoters for the kanamycin gene, which may not be deleted in this plasmid, are responsible for the transcription of the streptomycin A resistance gene.

Downstream of the streptomycin resistance gene is a region homologous to a region downstream of the *H. influenzae* R_{Rob} plasmid ROB-1 gene (Figure 7, bases 1849-2016 [Juteau & Levesque, 1990; GenBank accession number M64762]), encoding a β-lactamase gene (Figure 7). This is followed by a unique region of pLS88, and then a region homologous to the complement of a region upstream of the ROB-1 gene (Figure 7, bases 2338-2465 [Juteau & Levesque, 1990; GenBank accession number M64763]). What significance these sequences have, if any, is not known.

The kanamycin resistance gene sequence of pLS88 shows 99% homology to the transposon Tn*903*, but lacks the terminal inverted repeats of the transposon (Grindley & Joyce, 1980; Oka, et al., 1981). A comparison between the pLS88 and Tn*903* kanamycin resistance gene translation is given in Figure 8C.

The region of pLS88 between the kanamycin resistance gene and the sulfonamide resistance gene (Figure 7, bases 1-107 and 3898-4772) is not essential for plasmid replication or maintenance, as is shown by the deletion derivative pPC100 (Figures 3 and 7). Interestingly, this region contains a remnant of the RSF1010 *oriV* sequence, lacking only the three 20 bp repeats of the origin (Figure 7, bases 4095-4422). As well, some sequence homology exists in this region with pHD148 and pHD8.1 regions upstream of the sulfonamide resistance gene (Figure 7, bases 4423-4577, and Figure 13, bases 1785-1921).

2. Tn*903* AND TRANSPOSONS IN *HAEMOPHILUS*

The kanamycin resistance gene of pLS88 is homologous to the Tn*903* sequence, but lacks the inverted repeats which are necessary for transposition (Grindley & Joyce, 1980; Oka, et al., 1981). Why the inverted repeats are not present is not known: possibly, they interfere with plasmid function in the *Pasteurellaceae*. Most of the transposons (for example, Tn*5*, Tn*9*) except Tn*916* (Kauc & Goodgal, 1989; Holland, et al., 1992) and possibly Tn*2* have not been shown to transpose in the *Pasteurellaceae*, so it is possible that the terminal repeats are missing for a reason.

The promoters for transcription of the kanamycin resistance gene in pLS88 are not known. In Tn*903*, the promoters are thought, but not proven, to be located within the approximately 100 bases before the kanamycin resistance gene sequence (Grindley & Joyce, 1980; Oka, et al., 1981). Although pLS88 lacks the inverted repeats, the plasmid does contain some Tn*903* sequence (Figure 7, bases 3006-3081) upstream of the kanamycin resistance gene. Within this region, there are sequences characteristic of a transcriptional promoter (Rosenberg & Court, 1979) and a Shine-Dalgarno sequence (Shine & Dalgarno, 1974). It is possible that these sequences initiate transcription of the kanamycin resistance gene; if so, this promoter cannot be responsible for the expression of the streptomycin A resistance gene in pPC100, and a fourth, unidentified promoter is present in both pLS88 and pPC100 which can initiate transcription of the streptomycin A resistance gene.

3. ORIGIN OF REPLICATION

The sequence of the *ori* of pLS88 was not found to have homology with those of known origins. Through the creation of pPC100, the *ori* was isolated to a region 1060 bp in length, downstream of the streptomycin resistance gene (Figure 7, bases 1845-2675). This region has few repeat sequences (16 bp inverted [Figure 7, bases 1866-1907, with a 9 bp loop] and 11 bp direct [Figure 7, bases 2441-2462), one imperfect DnaA box (Figure 7, bases 2412-2422, inverted and complemented [Fuller, et al., 1984]) and two potential integration host factor (IHF) binding sites (Figure 7, bases 2497-2524 and 2583-2600). Included in the 1060 bases are the sequences homologous to regions flanking the *H. influenzae* ROB-1 β-lactamase gene and a small open reading frame (ORF; Figure 7, bases 2395-2675). It is not known whether the *H. influenzae* ROB-1 sequences are involved in replication.

Since both pLS88 and pPC100 contain the open reading frame (261 bp in length), the possibility that a plasmid-encoded replication protein may be involved in plasmid replication was explored. *In vitro* translation of both pLS88 anf pPC100 failed to show the production of a protein from the ORF (Figure 9). It is possible that a replication protein is encoded by the plasmid genome, but is produced in too little quantity or degrades too quickly to be detected by methods used in this study. Another possibility is that an RNA product may be involved in the replication process of pLS88.

The system of replication in pLS88 is unknown. The replication origin is small, yet capable of replication in a wide variety of bacterial hosts (Table 3). It does not appear to depend on any plasmid encoded replication proteins, a characteristic more commonly seen in narrow-host-range plasmid origins.

B. PLASMIDS pHD8.1 AND pHD148

1. SEQUENCE HOMOLOGIES WITH KNOWN PLASMIDS

Heteroduplex analysis between RSF1010 and pHD148 has been previously done (Albritton, et al., 1982). Results from the previous study, combined with the results from this study involving heteroduplexes between pHD148 and pHD8.1 (Figure 10), have highlighted two areas where gross differences exist between pHD148, pHD8.1, and RSF1010. These differences are in the areas of the RSF1010 *oriV* and streptomycin resistance genes, and between *repC* and the sulfonamide resistance gene (see Figure 2 for plasmid maps).

Heteroduplex analysis has suggested that the area corresponding to the RSF *oriV* region in pHD148 and pHD8.1 has been deleted. This has been confirmed by sequencing (Figure 12). However, further sequencing of both pHD148 and pHD8.1

has shown that *ori*-like sequences are present, but are found downstream of the *repC* gene (Figure 13, bases 59-496 and Figure 14). This "movement" of the *ori* has not affected the *oriT* site (Figure 12, bases 1388-1478), but two deletions around the *oriT* affect the promoter sequences P1, P2, and P3 identified in RSF1010 (Scholz, et al., 1989; Frey, et al., 1992) that drive the transcription of the *rep* and *mob* genes. Promoter sequence changes are small, and it is likely that promoter functions are not affected. The "moved" *ori* regions of pHD148 and pHD8.1 are highly similar to each other and to RSF1010, but not identical (Figure 14). The genes necessary for replication in RSF1010, *repA, repB,* and *repC*, are present in both pHD148 and pHD8.1.

Downstream of the *ori*-like sequence in pHD148 is a long sequence of unique DNA (the insertion loop, or IL; see Figure 2 and Figure 13, bases 517-1784) which contains a stem-loop structure (Figure 13, bases 346-572, at arrows). The stem is at least 34 bases in length, contains a high GC content, and has only one identified mismatched pair. The stem has sequence homology to the *ssi* sites of the RSF1010 *oriV*, which is interrupted by the IL. Because of the size and content of the stem, it has been difficult to sequence through the area. The size of the stem-loop is not accurately known, due to sequencing problems. If the sequence downstream of the stem-loop, slightly more than 1,200 bases, has a function, it is not known.

Downstream of the pHD148 insertion loop is a short sequence of DNA which is also present in pHD8.1 and pLS88, but not in RSF1010 (Figure 13, bases 1785-1922). The origin of this sequence is not known. This region is followed by DNA sequence homologous to the RSF1010 region upstream of the sulfonamide resistance gene, which contains the RSF1010 P5 and P6 promoters, thought to drive the transcription of the sulfonamide and streptomycin resistance genes in all four plasmids (Figure 13, bases 1923-2090).

The sulfonamide-streptomycin A intergenic regions of pHD8.1 and pLS88 have an identical 38 bp deletion fron the RSF1010 sequence (Figure 11), suggesting that pHD8.1 and pLS88 are more related to each other than either is to RSF1010. The streptomycin resistance genes are deleted in pHD148, but this plasmid has the 38 bases that pHD8.1 and pLS88 lack: interestingly, the crossover point for the deletion of the streptomycin resistance genes in pHD148 occurs immediately after these 38 bases (Figure 11).

The 150 bp immediately downstream of the pHD148 sulfonamide resistance gene is homologous to the sulfonamide resistance gene downstream region of the plasmid pGS05 (Rådström, et al., 1991). This sequence is not seen in pHD8.1, pLS88 or RSF1010. As well, there is homology between that pGS05 sequence and the insertion loop of pHD148, suggesting that the IL sequence of pHD148 may have originated from pGS05 (Rådström, et al., 1991). The region immediately upstream of the sulfonamide resistance gene in pHD148, pHD8.1, pLS88, RSF1010, and pGS05 are all homologous.

Figure 18 summarizes the interrelationships between the plasmids pHD148, pHD8.1, pLS88, RSF1010, and pGS05.

2. INCOMPATIBILITY AND THE ORIGIN OF REPLICATION

The plasmids RSF1010, pHD148 and pHD8.1 are incompatible, suggesting that their replication machinery is alike. Therefore, it is likely that the *ori*-like sequences of pHD148 and pHD8.1 discussed above (Figure 14) behave in a manner similar to the RSF1010 *oriV*. However, pHD148 and pHD8.1 are capable of being stably maintained within the *Pasteurellaceae*, and RSF1010 is not.

The ability of both pHD148 and pHD8.1 to replicate within the *Pasteurellaceae* may be related to the movement of the *ori* sequence from upstream of the *oriT* to downstream of *repC* (Figures 12, 13, and 18). Other researchers, who have confirmed the instability of RSF1010 within the *Pasteurellaceae*, have also demonstrated the stable replication of RSF1010 minimum replicons in both *Actinobacillus pleuropneumoniae* and *Pasteurella haemolytica* (Frey, 1992). Exactly why these alterations in structure allow stable replication within the *Pasteurellaceae* is not understood.

Figure 18. Physical maps of plasmids RSF1010, pHD148, pHD8.1 and pLS88.

The map at the top represents the entire RSF1010 plasmid linearized at the *Pst*I site immediately upstream of the sulfonamide resisrance gene. The locations of genes and structures are taken from Scholz, et al., 1989.

The maps at the center and bottom represent the entire of pHD8.1, pHD148, and pLS88, linearized as per RSF1010. The locations of genes and structures are from Willson, 1989, or were determined in this study. The size from 0 to 8.8 kb is indicated by a scale at the bottom.

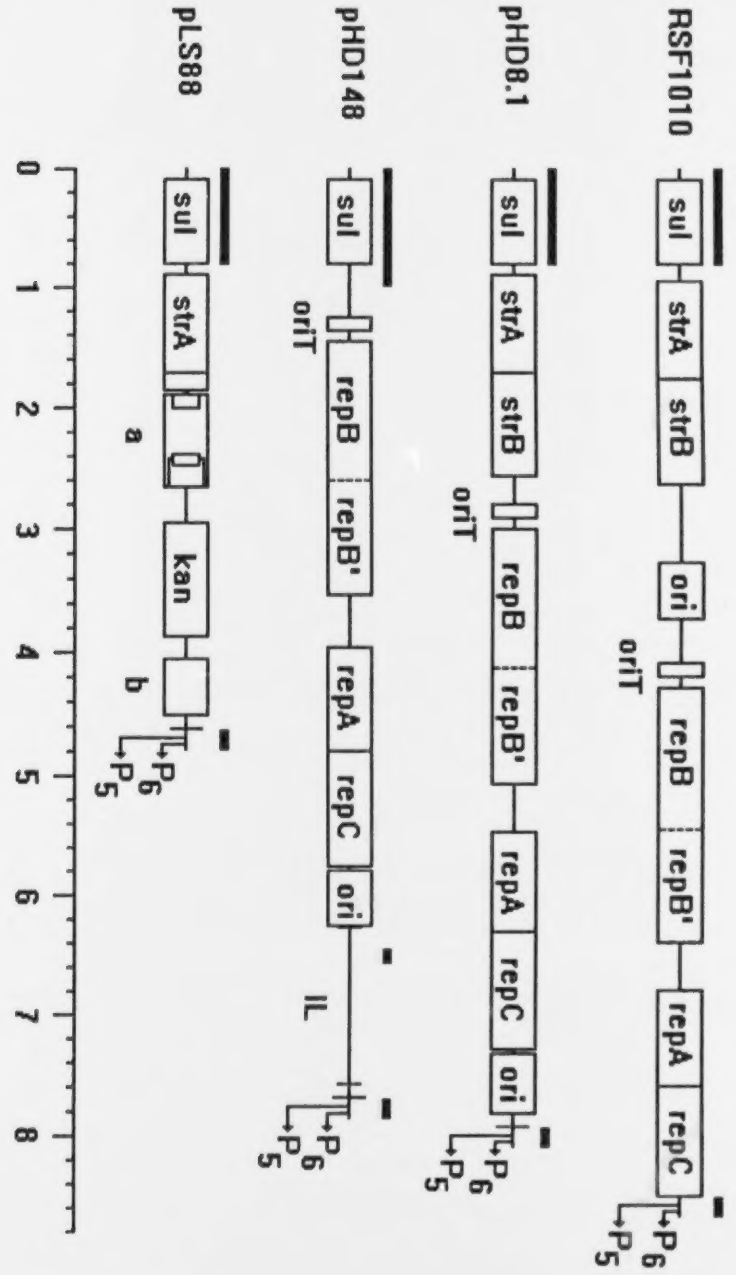
Box (a) represents the *ori* of pLS88. The smallest boxes within the region of the origin represent the sequences homologous to regions before and after the *H. influenzae* ROB-1 β-lactamase gene. The larger box represents the ORF.

Box (b) represents the region of pLS88 homologous to the incomplete RSF1010 *oriV* region, which lacks the three 20 bp direct repeats.

Between box (b) and the end of pLS88 is a line, which indicates the end of a region of DNA homologous to pHD8.1 and pHD148 (see lines on these plasmid maps as well) but not RSF1010. Downstream of this line is sequence common to all four plasmids.

The black lines over areas of the four plasmids indicate regions of homology with the known sequences of plasmid pGS05 (Rådström, et al., 1991).

Figure 18. Physical maps of plasmids RSF1010, pHD148, pHD8.1 and pLS88.


RSF1010
pHD8.1
pHD148
pLS88
sul
strA
strB
ori
oriT
repB
repB'
repA
repC
P6
P5
sul
strA
strB
oriT
repB
repB'
repA
repC
ori
P6
P5
sul
oriT
repB
repB'
repA
repC
ori
IL
P6
P5
sul
strA
a
kan
b
P6
P5
0
1
2
3
4
5
6
7
8

C. PLASMID pDM2

1. SEQUENCE HOMOLOGIES WITH KNOWN PLASMIDS

The RSF0885 region of the plasmid has been previously discussed (Figures 4 and 5). The sequence of the 4 kb *Bam*HI fragment of p2265 that comprises the remainder of the plasmid pDM2 was generated by primer extension in both directions out from the chloramphenicol resistance gene sequence of pSa, encoding a type II chloramphenicol acetyl transferase (Foster & Shaw, 1973; Murray, et al., 1990), and is given in Figure 16.

The known pSa sequence bordering the chloramphenicol resistance gene (Murray, et al., 1990) is present in the *Bam*HI insert from p2265 found in pDM2: the sequence is homologous between positions 1530-2498 (Figure 16). Between the two direct repeats and the sequence homology with pSa are two groups of short repeat sequences, two 11 bp direct and three 18 bp direct repeats (Figure 16, bases 1200-1380). What function these serve, if any, is not known. It is also not known if the sequence including the short direct repeats is present in pSa, or if that region was generated by a separate recombinational event in p2265. The function of the sequence in p2265 (and pDM2) downstream of the region homologous to pSa and upstream of the third, inverted repeat is likewise unknown. This region is not present in pSa, as the 3' region of homology between the p2265 *Bam*HI insert and the pSa sequence occurs within the known pSa sequence.

2. Tn*10* AND TRANSPOSONS IN *HAEMOPHILUS*

The three long repeat sequences present at the border of the 4 kb *Bam*HI insert contained in pDM2 form part of a larger structure including tetracycline resistance genes in several large conjugative plasmids, including p2265, that can be found in *H. influenzae*. These plasmids share an almost identical core, with the antibiotic resistance genes located on discrete, translocatable genetic elements (vanKlingeren, et al., 1977; Spies, et al., 1983). Research on the occurrence of multiple plasmid-linked antibiotic resistance have provided evidence for the existence of combined transposons, containing an ampicillin or chloramphenicol resistance gene inserted into a tetracycline transposon, which serves as a vehicle for transposition (Spies, et al., 1983).

Another mechanism used in the adaptation to increased antibiotic concentrations has been reported in *H. influenzae*: it has been observed that tetracycline transposons as well as combined tetracycline and chloramphenicol transposons may be amplified, producing multiple copies in succession (Jahn, et al., 1979; Spies, et al., 1983). Similar phenomena have been observed on certain plasmids of *Streptococcus faecalis* (Clewell, et al., 1975), *Proteus mirabilis* (Rownd & Mickel, 1971), and *E. coli* (Schmitt, et al., 1979). In these cases, the amplifiable units are bound by direct repeats of various lengths, providing homology for recombination.

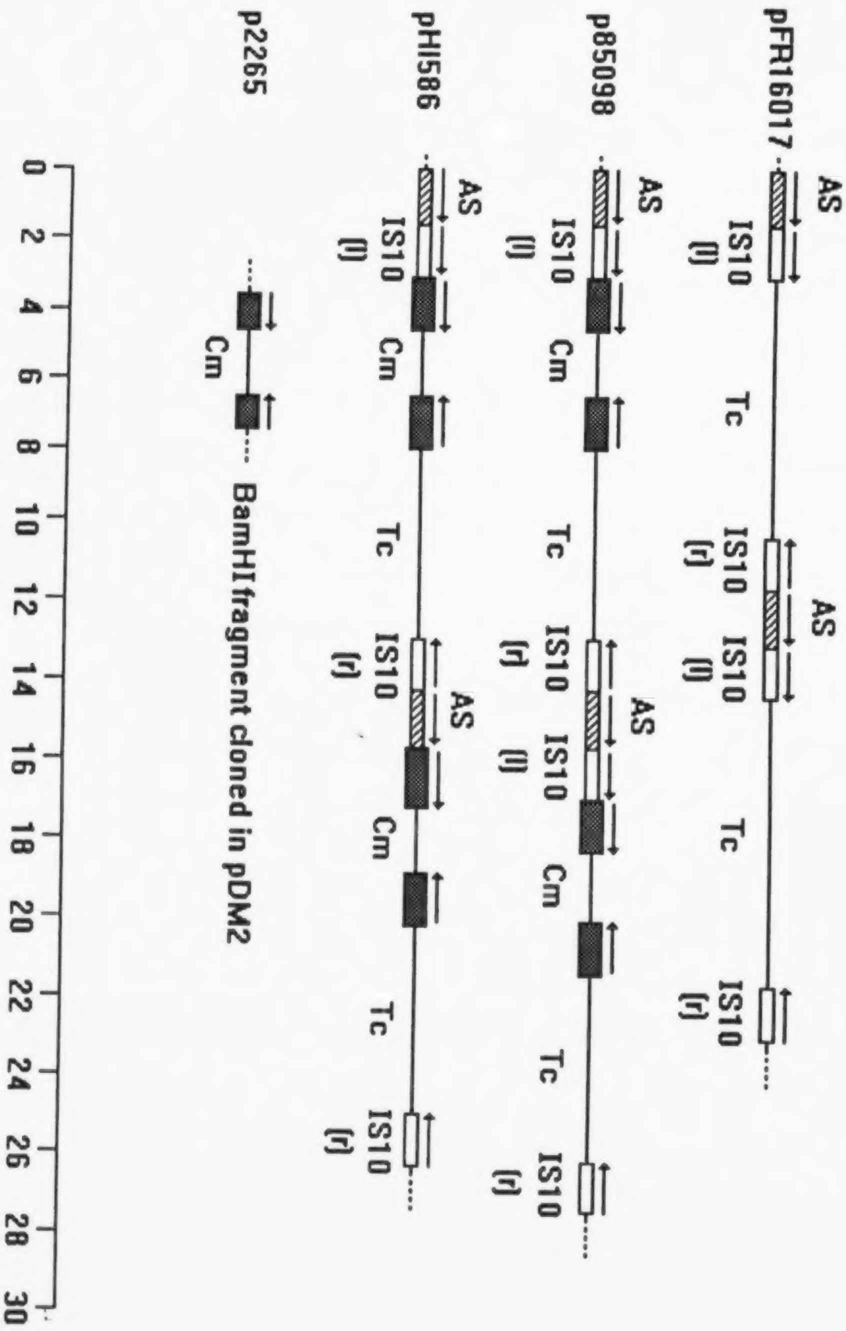
p2265 is thought to contain combined tetracycline and chloramphenicol transposons, and the *Bam*HI fragment of p2265 contained in pDM2 is likely to be a part of one. In the study by Spies, et al., digestion of plasmids containing combined transpsons by *Bam*HI cleaved out the chloramphenicol resistance gene; moreover, the cleavage was symmetrical within the inverted repeats bounding the gene, cleaving approximately 500 bp apart (Spies, et al., 1983). In the p2265 fragment, inverted repeats approximately 500 bp in length and bounded by *Bam*HI sites were found (Figure 16, bases 1-445,446-886 and 3448-3969, and Figure 19). It is possible that, given such a typical transposon structure, this may represent a chloramphenicol transposon in *Haemophilus*. However, no such structure studied thus far has been shown to transpose independently of the Tn*10* element, and amplification of the chloramphenicol resistance gene was not observed with increasing concentrations of chloramphenicol (Spies, et al., 1983).

Figure 19. Physical maps of the 4.1 kb *Bam*HI fragment of p2265 and the large Tn*10*-chloramphenicol resistance plasmids.

The top three maps are the amplifiable units of large conjugative *Haemophilus* plasmids containing resistance transposons (Spies, et al., 1983). IS10 (l) and (r) are the inverted insertion sequences that border the transposon Tn*10*. AS, represented by hatched areas, are "amplification sequences" which are contained within each amplifiable unit. Cm and Tc represent the approximate positions of the chloramphenicol and tetracycline resistance genes. Shaded areas represent the repeat sequences of the chloramphenicol resistance gene. Arrows indicate the direction of repeated sequences. The size from 0 to 30 kb is indicated on the bottom.

The plasmid p2265 *Bam*HI fragment containing the chloramphenicol resistance gene is represented in the map on the bottom. The organization of the structures surrounding it are not known. The sequence of the fragment is in Figure 16.

Figure 19. Physical maps of the 4.1 kb *Bam*HI fragment of p2265 and the large Tn*10*-chloramphenicol resistance plasmids.


pFR16017
p85098
pHI586
p2265
AS
IS10
(l)
Tc
IS10
(r)
IS10
(l)
IS10
(r)
Cm
BamHI fragment cloned in pDM2
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30

D. PLASMIDS pPW87 AND pPW88

1. SEQUENCE HOMOLOGIES WITH KNOWN PLASMIDS

The sequence of the 3.4 kb *Pst*I fragment of pSa contained in pPW87 has been determined and is found in Figure 17. What is interesting in the sequence bordering the chloramphenicol resistance gene is its homology to the sequence bordering the chloramphenicol resistance gene of pDM2. Homology exists between the pPW87 insert sequence at positions 781-2080 and the pDM2 insert sequence at positions 1122-2498, except in the region of pDM2 containing the short direct repeats. In the pPW87 sequence, there are two deletions, one 28 bp in length, and the other 33 bp in length, that occur in the regions in pPW87 corresponding to the short repeats in pDM2 (Figure 20). Since part of the sequence of the two outer longer repeats are present in pPW87, it is likely that these repeats were deleted from the pPW87 sequence. It is not known whether these repeats were deleted after the *Pst*I fragment was cloned into pLS88, or if they have been deleted in pSa prior to cloning.

Downstream of the chloramphenicol resistance gene of pPW87 are two approximately 400 bp direct repeats, each containing a *Xho*I site (Figure 17, bases 2486-2882 and 2883-3277). In pPW88, containing the pSa* insert, there are three

Figure 20. Physical maps of plasmid insert sequences from pDM2 and pPW87, and the corresponding sequence in pSa.

The map at the top represents the *Bam*HI fragment of plasmid p2265, found in pDM2. The large open arrows represent the terminal inverted repeat sequences. The small black arrows represent the two groups of short direct repeats seen in the insert sequence.

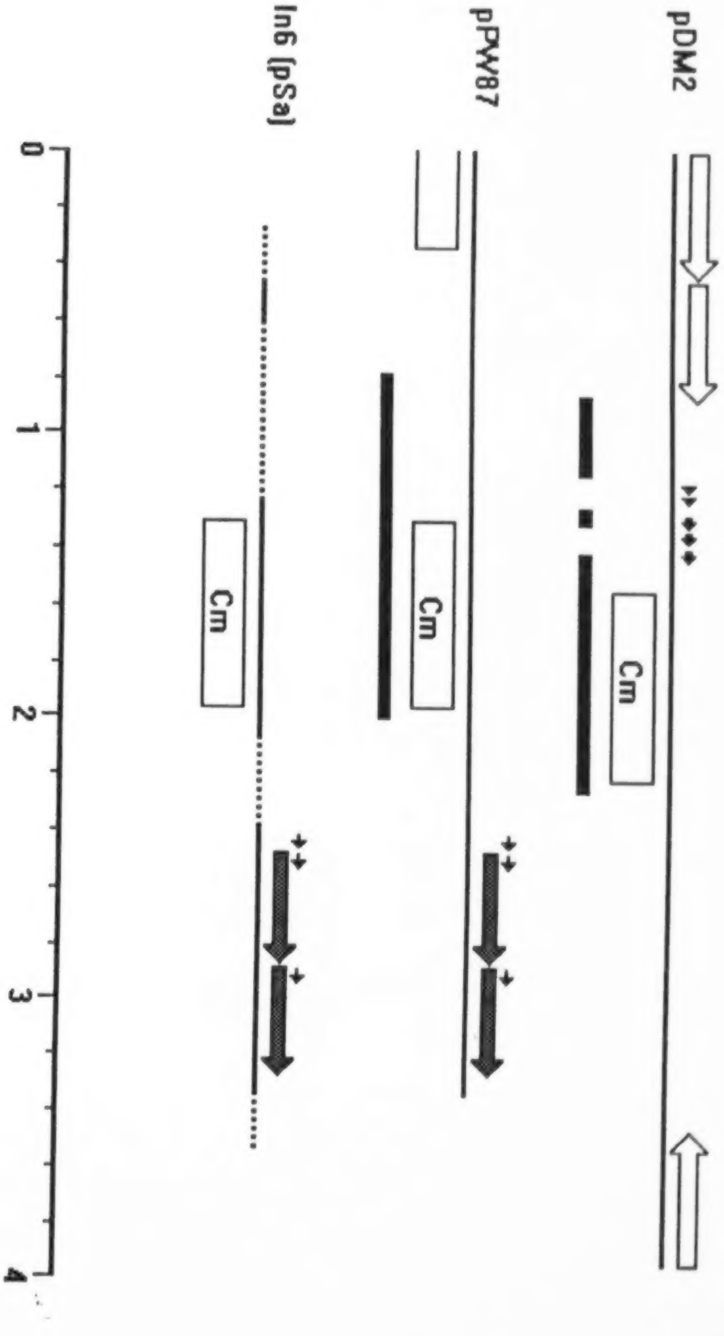
The map in the middle represents the *Pst*I fragment of plasmid pSa, found in pPW87. The large grey arrows represent the long direct repeat sequences. The small black arrows represent the short duplicated sequence at the 5' end of the repeats. The open-ended box represents the incomplete sulfonamide I resistance gene found in the integron of pSa, In6.

The map at the bottom represents the known published sequences of In6 from pSa (solid black line; Murray, et al., 1990; Stokes, et al., 1993) separated from regions not yet sequenced (dotted lines), lined up against the pPW87 insert sequence.

The thick, solid black lines indicate regions of homology between the pDM2 and pPW87 insert sequences.

The size from 0 to 4 kb is indicated on the scale at the bottom.

Figure 20. Physical maps of plasmid insert sequences from pDM2 and pPW87, and the corresponding sequence of pSa.


pDM2
pPW87
In6 (pSa)
Cm
Cm
Cm
0
1
2
3
4

direct repeats, each containing a *Xho*I site (Figure 6). It is possible that these repeats may be responsible for the deletion events observed in these plasmids.

2. INTEGRONS

The pSa insert sequence is part of a larger structure in pSa called an integron (In). Integrons are DNA elements encoding a site-specific recombination system capable of acquiring genes at a specific site. Typically, integrons consist of two highly conserved DNA sequences, designated the 5'- and 3'-conserved segments, flanking a variable insert region which encodes one or more antibiotic resistance genes (Stokes & Hall, 1989). The 5'-conserved segment encodes a DNA integrase and the 3'-conserved segment includes a sulfonamide resistance gene (*sul*I). The nature, number, and order of genes in the variable region varies widely. These genes are inserted as cassettes, and each cassette includes a gene coding region, a 59 base element located at the 3' end of the gene (excluding the last seven bases), and a seven base core recombination site at the 5' end of the gene (Stokes, et al., 1993). The 59 base sequences act as sites for the site-specific recombination events catalyzed by a DNA integrase, the gene for which is present in the 5'-conserved segment

(Collis & Hall, 1992*a*; 1992*b*; Collis, et al., 1993; Stokes, et al., 1993). Recombination occurs within or beside a GTT triplet found in all seven base core sites. These inserts have the features of gene cassettes, and encode a single open reading frame. The variable regions contain very little noncoding DNA (Stokes, et al., 1993). Maps of integron structures are found in Figure 21.

Two atypical integrons have been described. In7 is found in a 120 kb conjugative IncC plasmid (Stokes, et al., 1993). In6 is found in the plasmid pSa (Stokes, et al., 1993; Ireland Valentine, 1985). These integrons are unusual in that they contain two copies of the sulfonamide resistance gene, and therefore part of the 3'-conserved segment on the 5' end (Stokes, et al., 1993).

The chloramphenicol resistance gene in pPW87 and pPW88 is from the region of In6 between the two 3'-conserved segments. Normally, gene cassettes inserted into integrons contain little more than the gene itself. Where the direct repeats observed in the *Pst*I fragment originated from is unknown. The repeats were not acquired from the same site as the chloramphenicol resistance gene: In7, which is remarkably similar to In6, contains the sequence of one repeat, but does not contain a chloramphenicol resistance gene (Stokes, et al., 1993). It is likely that the one repeat sequence seen in In7 was tandemly duplicated in In6, along with a further direct duplication of the first 28 bases (Figures 16 and 20). In

Figure 21. Physical maps of the plasmid pPW87 and Int6.

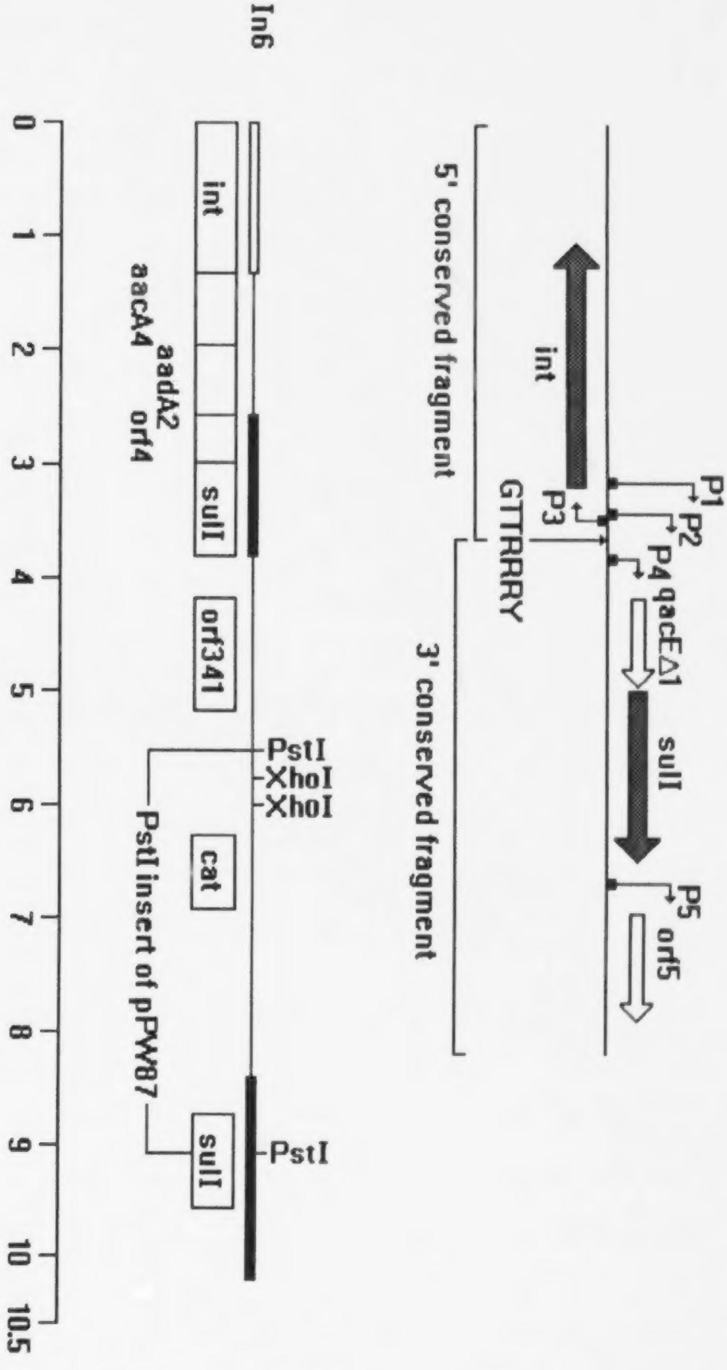
The map at the top illustrates the general structure of integrons. The location and orientation of the different promoters are shown. The arrow marked *int* denotes the gene for the integrase. The product of *qacE*Δ1 determines resistance to ethidium bromide and quaternary ammonium compounds. The putative product of orf5 is similar to the puromycin acetyltransferase of *Streptomyces alboniger*. The sequence GTTRRRY is the integron's crossover point for integration of gene cassettes (Stokes & Hall, 1989; Hall et al., 1991; Stokes, et al., 1993; Collis & Hall, 1992).

The map on the bottom represents In6 from pSa (Stokes, et al., 1993; Ireland Valentine, 1985). The 5'-conserved segment is shown as a hollow line and the 3'-conserved segments are shown as thick black lines, illustrating the duplication of part of the 3'-conserved segment. The *int, sulI* and *cat* genes encode the integrase, and sulfonamide and chloramphenicol resistance proteins. The predicted product of orf4 is closely related that of *qacE*Δ1. The genes *aadA*2 and *aacA* encode spectinomycin resistance and kanamycin and gentamycin resistance, respectively (Stokes, et al., 1993). The orf341 gene shows some homology to a noncoding ORF in RSF1010.

The position of the *Pst*I fragment present in pPW87 is highlighted at the bottom. In pPW88, three *Xho*HI sites have been identified. The sequence of pPW87 is in Figure 17.

The size, from 0 to 10.5 kb, is indicated at the bottom.

Figure 21. Physical maps of the plasmids pPW87 and pPW88 and In6.


P1
P2
P3
P4
P5
int
qacEΔ1
sulI
orf5
GTTRRRY
5' conserved fragment
3' conserved fragment
In6
int
aadA2
aacA4
orf4
sulI
orf341
cat
PstI
XhoI
XhoI
PstI
sulI
PstI insert of pPW87
0
1
2
3
4
5
6
7
8
9
10
10.5

pSa*, a further duplication occurred, giving rise to three direct, tandem repeats.

E. SUMMARY OF GENETIC ANALYSIS IN THE *PASTEURELLACEAE*

1. THE PLASMID pLS88

The 4.8 kb plasmid pLS88 has been fully sequenced and well described. Its usefulness as a potential vector stems from its small size, multiple antibiotic resistance genes, and broad host range. The simplicity of the plasmid structure is quite appealing.

pLS88 encodes resistance to streptomycin, sulfonamide, and kanamycin. The DNA sequences for the resistance genes are highly homologous to antibiotic resistance genes from both RSF1010 (*sulII* and *strA*) and Tn903 *(kan)*. Despite the sequence homology with RSF1010, pLS88 is from a different incompatibility group than RSF1010.

The broad-host-range of pLS88 is indicated by the ability of the plasmid to confer resistance to streptomycin and kanamycin within members of the *Enterobacteriaceae,* the *Pasteurellaceae*, and the *Pseudomonaceae.* The acquisition of the antibiotic resistant phenotype by these bacteria indicates that promoters for the resistance genes are recognizable by the transcriptional apparatus, and that the origin of replication can function within these organisms.

The replication mechanism of pLS88 remains to be described. The origin of replication is small, and does not appear to depend on any plasmid encoded *rep* proteins. It is possible that a replication protein is encoded by the genome, but is produced in too little quantity or degrades too quickly to be detected by methods used in this study. Another possibility is that an RNA product may be involved in the replication process of pLS88. Given the small size of the *ori* region, the broad nature of the host range is surprising.

The antibiotic resistance genes in pLS88 have unique restriction sites that make the plasmid a good cloning vector. The sulfonamide resistance gene has two *Pst*I sites that can be used to replace *sulII* with foreign DNA. The kanamycin resistance gene has unique cloning sites for *Cla*I, *Hind*III, *Pvu*I, *Sma*I and *Xho*I. The streptomycin resistance gene has a unique cloning site for *Bgl*I. These restriction sites will allow insertion of foreign DNA with easy selection for recombinant plasmids.

2. THE PLASMIDS pPW87 AND pPW88

The recombinant plasmids pPW87 and pPW88 have also been fully sequenced. The plasmids contain genes encoding streptomycin, kanamycin (from pLS88) and chloramphenicol (from pSa or pSa*) resistance. The chloramphenicol resistance gene has a unique *Pvu*II site for cloning.

The pSa and pSa* inserts containing the chloramphenicol resistance gene have repeat sequences which may explain the deletion events occurring in pPW87 and pPW88 under different antibiotic selection. It may be possible to use these plasmids as partial suicide vectors, as the chloramphenicol resistance gene is deleted when selecting for streptomycin resistance in both *H. influenzae* and *A. pleuropneumoniae* (Willson, 1989). In this way, DNA cloned into the chloramphenicol resistance gene can be released, through recombination, from pPW87 or pPW88 *in vivo*. If *Haemophilus* or *Actinobacillus* chromosomal DNA was cloned into the plasmid chloramphenicol resistance gene through manipulation in *E. coli*, homologous recombination can result in insertion mutants in *Haemophilus* or *Actinobacillus*. This will allow for observation of the effects of insertion mutations on specific areas of the *Haemophilus* or *Actinobacillus* genome.

3. THE PLASMID pDM2

The entire sequence of pDM2 has now been determined. Its use as a broad-host-range shuttle vector is already known. It is capable of replication in both *Haemophilus influenzae* and *E. coli* (McCarthy, et al., 1982). The ampicillin marker has unique restriction sites for *Pst*I and *Pvu*I and the chloramphenicol resistance marker has a unique restriction site for *Sma*I (McCarthy, et al., 1982).

The p2265 insert containing the chloramphenicol resistance gene shows homology to the pSa insert in pPW87 and pPW88, suggesting a common origin. The long repeat sequences of both inserts are different in sequence and orientation and are not derived from the same origin. Because of the deletion of the short direct repeats of the p2265 insert sequence as seen in the pSa insert sequence, it is more likely that the pSa sequence is decendent from the p2265 sequence (with subsequent deletion of the short direct repeats) than *vice verca*. The long repeats of the pSa sequence may have already been present prior to the insertion of the chloramphenicol resistance gene into the integron.

The p2265 insert containing the chloramphenicol resistance gene is worth further study as a possible transposon in *Haemophilus*. The study of the combined tetracycline and chloramphenicol transposon in p2265 should continue to this end.

4. THE PLASMIDS pHD8.1 AND pHD148

Plasmids pHD8.1 and pHD148 have a limited use, in their current form, as vectors. Although the host range of these plasmids is quite broad, they lack multiple markers useful in the easy selection of clones from a mixed population. The addition of more markers to these plasmids through cloning is possible; however, the increasing size of the plasmids will begin to be of concern. As well, they are incompatible with RSF1010-based vectors which are commonly used for genetic analysis.

The replication mechanism of pHD148 and pHD8.1 has been discussed, and appears similar to the mechanism of RSF1010 replication. The manner by which these plasmids are able to replicate in the *Pasteurellaceae* while RSF1010 cannot is puzzling, and work remains to be done concerning what inhibitory structures or mechanisms are present in RSF1010 that are deleted or inactivated in the movement of the replication origin to the sites in pHD8.1 and pHD148, as well as in the RSF1010 minimum replicons, which lack little more than the sulfonamide and streptomycin resistance genes.

The insertion loop in pHD148 could have originated from pGS05 through recombination. The whole sequence of the pHD148 insertion was not completed due to sequencing problems through the stem-loop structure. The sequence of pGS05 in this region has also not been fully determined. It would be interesting to see the full structure of the completed sequence, and the possible effect this structure could have on plasmid functions.

This study presents the characterization of several potential vectors for use in the genetic analysis of members of the *Pasteurellaceae*. The plasmids pLS88, pPW87/88 and pDM2 will be useful as broad-host-range shuttle vectors in the *Pasteurellaceae* and the *Enterobacteriaceae*. The plasmids pHD148 and pHD8.1, while interesting, are of limited use as vectors in their present form. Given the medical and economic importance of this bacterial family, and that no commercial vector systems exist for use in the *Pasteurellaceae*, it is important to continue the characterization and analysis of these plasmids, including replication mechanisms, promoter sites and structure, and recombinational events, to better understand their potential as shuttle vectors.

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